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THE VIRUS, THE NERVE CELL, AND PARALYSIS

A STUDY OF EXPERIMENTAL POLIOMYELITIS IN THE SPINAL CORD¹

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CONTENTS

Material	4
Plan of Study	5
Description and definition of clinical stages	6
Description and definition of grades of paralysis	8
Description of findings	9
I Cytopathological Changes in Motor Nerve Cells	9
Nissl substance	
Mitochondria	
Neurofibrils	
Axons	
Nuclei	
Criteria of necrobiosis in motoneurons	
II The Sequence of Pathological Changes in the Anterior Horn, with a Statistical Study of Injury and Recovery Stages in Motor Nerve Cell Populations	18
Pathological characteristics of successive clinical stages	18
The preparalytic period	
The first day of paralysis	
Second and third days	
Four to six days	
Seven to twelve days	
The third week	
35 to 49 days	
Convalescent stage	
Summary of quantitative data Dynamic aspects of the infection and recovery of the motoneuron population	28
Determination of the distribution of the normal motoneuron population	

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Changing proportions of normal, abnormal, and destroyed cells in the total motoneuron population	
Changing proportions of normal cells, and abnormal stages, with respect to the total number of remaining cells	
III Damage and Recovery of the Nerve Cell Population as Related to Motor Paralysis and Recovery	44
The role of motoneuron destruction	
The role of reversible motoneuron injury	
The role of distribution of cell injury and destruction	
Distribution of lesions in non-paralytic cases and in monkeys infected with "mild" strains	
Discussion	58
Cytopathological changes in motoneurons	
Comparison of humans and chimpanzees with rhesus monkeys	
Comparison of poliomyelitis with other neurotropic virus diseases	
Statistical findings	
Relation of changes in motoneurons to paralysis and recovery	
Degree of cell injury in relation to functional state, and to virus multiplication	
Persistent perivascular lesions	
Summary	68
References	70

INTRODUCTION

Published observations on the histopathological changes in the spinal cord in poliomyelitis are so numerous that it would seem little can be added to what has been reported. The complexity of the problems of pathogenesis, however, has resulted in contradictory interpretations of certain findings, and has left many points unsettled. It was felt that some of the important points remaining at issue perhaps could be investigated more satisfactorily by means of an approach which attempts to correlate pathological processes in chronological sequence, with changes in level of virus activity on the one hand and changes in clinical function on the other. It was felt that such an approach requires at least approximate quantitative data at all three levels of observation, and could therefore be essayed only in the experimental animal. The findings and interpretations to be reported are by no means complete, and because of the nature of the material the quantitation of the variables involved often leaves much to be desired. It is hoped, nevertheless, that the results may be useful as a rough scaffold

folding for the tasks of planning and interpretation of studies at the chemical or virus level, and of improving our understanding of cellular changes in relation to paralysis and recovery. The basic question of the time sequence of rise and fall of virus concentration in the infected rhesus spinal cord has not received close attention except by Brodie ('33) and Bodian and Cumberland ('47). The findings in these studies have served as an important background for orientation in the work reported here.

The first objective of this study was to determine the sequence of histological changes which occurs in the spinal cord from the period of onset of pathological changes to the period of functional recovery in poliomyelitis, and to establish relatively constant features of the cytopathological process in quantitative detail. The changes in the motoneuron population were studied particularly, since the motoneurons bear the brunt of the pathological effects and probably constitute the principal source of substrate for virus proliferation. In addition, their destruction is the principal cause of motor paralysis and of muscle atrophy which follows the paralysis. The population unit selected for study was the motoneuron group supplying a single extremity, arm or leg, and consisting of about 14,000 motor nerve cells. This population unit was selected because it can be identified spatially much more accurately than smaller units supplying individual muscles or muscle groups, because it is relatively compact in distribution, and because the field it supplies, the arm or leg, is a functional unit not too difficult to assess in terms of degree of functional loss. In fact, therefore, the motoneuron population supplying a limb, plus the limb itself, can be considered as a model for the disease in the whole organism, undergoing the characteristic ebb and flow of the disease processes in all their ramifications.

Although important contributions to an understanding of the pathological events of the acute stage, and especially the preparalytic period, have been made by Hurst ('29) and by Pette, Demme, and Kornyei ('32) in their experimental studies, quantitative studies of the sequence of cellular changes in a close series of infective stages have been made only by Covell ('32). In his excellent study, unfortunately, Covell failed to appreciate that changes in cytoplasmic Nissl substance were sufficiently distinctive so that they could be used as a quantitative

measure of both the degree of injury and of recovery of nerve cells. Moreover, as he was aware, degenerative changes were overemphasized in his analysis, since all his animals had the prostrating type of paralysis produced by the Rockefeller Institute MV virus.

MATERIAL

Rhesus monkeys were used to enable study of changes from the earliest period of pathological effect to late stages of recovery, and to permit more satisfactory fixation and staining of tissues than is possible in human material. In all, some 50 rhesus monkeys killed from a few days to one year after inoculation with several "strains" of virus of recent human origin were studied quantitatively, and many others were available for qualitative comparisons. These monkeys had been examined in considerable clinical detail in most cases before they were killed, to determine the time of onset of symptoms, the degree of weakness, and the extent of recovery. Motor function of animals was determined by inspection of running, jumping and climbing performance in large cages, by hand examination of individual extremities to determine asymmetry of power of flexion and extension at joints, and by palpation to detect abnormalities of tone and atrophy. In most cases daily examinations were made during the acute period of the disease, with weekly, and later monthly, examinations during the convalescent period. In some animals, voluntary muscle performance was compared with the response to faradic stimulation of nerve points and exposed peripheral nerves at the time of autopsy.

After final clinical examination, animals were anesthetized with ether, and perfused through the aorta with 50 ml of normal saline followed by 1 to 2 liters of 10% formol containing 1% acetic acid. Some animals were fixed with 10% neutral formol, or with Regaud's fluid, for better study of mitochondria and inflammatory changes. The fixation of tissues by vascular perfusion of the living anesthetized animal was of great value in eliminating most of the postmortem artifacts seen in material fixed with the usual immersion technique. The cervical and lumbosacral spinal cord enlargements were removed carefully, and the four segments contributing principally to the innervation of upper and lower extremities were prepared for paraffin embedding. In the cervical region the cord segments regularly sectioned

were the 6th, 7th, and 8th cervical, and the 1st thoracic (C6, C7, C8, T1), in the lumbosacral region the 4th, 5th, 6th, and 7th lumbar (L4, L5, L6, L7). When the lumbar plexus was pre- or post-fixed, the four segments which had the largest spinal roots were considered for formal purposes as the 4th to 7th lumbar. Complete serial sections in paraffin were cut at 15μ and stained with gallocyanin, according to Einarson's method ('32), or alternately with gallocyanin, haematoxylin-eosin-azure, and activated protargol (Bodian, '36). Mitochondria were studied in 7μ sections of Regaud material, stained with iron-alum haematoxylin.

Serial sections of spinal cords from 10 human cases and 4 chimpanzee cases of poliomyelitis were available for comparison. Material from monkeys infected with Western equine encephalomyelitis virus, and with neurotropic yellow fever virus (17D strain), was also used for certain comparative purposes.

PLAN OF STUDY

A preliminary survey of all the material available, including chimpanzee and human spinal cords, established the essential similarity of the pathological process in all three primate species during the period of progressive paralysis and later. The earliest changes of the pre-paralytic period were available only in the rhesus monkey.

Attention was limited to the changes in the lateral cell columns of the anterior horn of the spinal cord of the brachial and lumbar enlargements, so that histopathological events might be correlated in time and in degree with changes in muscle function of each extremity. It was felt that only in this way was it possible to deal with the dynamics of the disease process. Because of the mixture of stages of cell change found in any one specimen, it was necessary to establish the precise sequence of events by statistical means, so that much time was devoted to this end. As a routine, 50 to 60 sections, out of a total of about 1200 sections in each brachial or lumbar enlargement, were subjected to cell counts. These sections were regularly spaced throughout the cord segments involved, so that 10 to 15 sections were examined as a sample of each of the four principal segments contributing to the brachial and lumbar plexuses, respectively. These segments in the brachial region are C6 to T1 inclusive, and in the lumbar region L4

to L7, inclusive. Although C5, T2, and S1 contribute to their respective limb plexuses, they were not usually studied. It was felt that the status of the motoneuron population of the four principal segments was an adequate indicator of the function of the innervated limb, so that study of the marginal segments would add tedium without much increase of accuracy. In all material only cells containing a recognizable nucleus and nucleolus were counted.

DESCRIPTION AND DEFINITION OF CLINICAL STAGES

For the purposes of this study it was necessary to define carefully the clinical stages of the disease in experimental animals, so that accurate correlations could be made between the clinical events, histopathological changes, and the rise and fall of virus in infected nervous tissue. Table 1 indicates the stages with extremes of time range observed in animals in this series. Other animals in this laboratory have exhibited incubation periods as short as 3 days and as long as 54 days. The *incubation stage* will be defined as the phase following inoculation and preceding clinical signs. This phase is subdivided into a *latent period* during which no virus or lesions can be detected in the central nervous system (CNS), and an early preparalytic or *presymptomatic period*, during which no symptoms are apparent, but during which virus and cell changes can be found in the CNS near the point of inoculation. The onset of fever, when it occurs, opens the following, or *acute stage*, beginning with the late preparalytic period. This period precedes detectable flaccid paralysis, and is characterized by generalized symptoms such as fever, tremor, spasticity and ataxia (Romer, '11, Harmon, et al, '31, Pette, et al, '32, Bodian and Cumberland, '47).

In the experimental animal, as in man, symptoms may or may not be present preceding paralysis. With the virus strains generally employed in experimental work, the preparalytic period usually covers an interval of about one day, but not infrequently two or three days. With small doses of virus, or with "mild" strains, the preparalytic period may be prolonged to five or more days, during which period fever, tremor, and clasp-knife spasticity may be present in varying degrees. For purposes of definition, it is to be noted that, irrespective of its duration, the preparalytic period opens with the critical phase of

beginning virus activity It is obvious that the termination of this period is arbitrarily defined Since extensive lesions of anterior horn cells may occur before muscle weakness is clinically recognized, the onset of the *period of increasing paralysis* is not sharply demarcated In terms of pathological histology, moreover, the latter period is simply

TABLE 1
Stages of Poliomyelitis in Rhesus Monkeys

	DURATION IN DAYS		CLINICAL SYMPTOMS	VIRUS	PATH CHANGES
	Mean	Range			
<i>Incubation Stage</i>					
Latent Period	8	3 to 22	0	0	0
Early Preparalytic Period	1		0	+	+
<i>Acute Stage (febrile)</i>					
Late Preparalytic Period	3	1 to 5	+	+ to 4+	+
Period of Increasing Paralysis	3	1 to 5	+	4+ to +	+ to 4+
<i>Subacute Stage (Early Recovery Period)</i>	21	15 to 40	+	+	+
<i>Convalescent Stage (Period of Continued Recovery)</i>	60	30 to 300	+	-	+
<i>Chronic Stage (No Further Recovery)</i>					

TABLE 2
Time Course of Paralysis, Atrophy, and Recovery in Rhesus Monkeys

	PERIOD OF DISEASE AFTER ONSET OF PARALYSIS	
	Mild Cases	Severe Cases
Height of Paralysis	1 to 3 days	2 to 5 days
Onset of Atrophy		2 to 3 weeks
Beginning Recovery of Power	1 to 3 weeks	1 to 2 months
Limit of Increase of Power and Muscle Size	1 week to 3 months	3 months to 1 year

a continuation of the preceding one, so that both periods represent the phase of progressive degradation of nerve cell functions due to virus activity

The *period of increasing paralysis* is followed by a *period of early recovery*, which, according to pathological criteria, is a phase of neuron recovery, and is usually largely completed about three to six weeks after onset of paralysis After this time, remaining nerve cells in the spinal

cord are essentially normal in appearance in all cases, as Hurst ('29) found in his single recovered animal, so that further functional recovery must be referable to other recovery and compensation changes in the CNS and in muscle. These changes occur in the *convalescent stage* and are followed by a phase of no further recovery, the *chronic stage*. Table 2 shows the time course of some of the critical points in the sequence of paralysis, atrophy, and recovery.

In defining the stages of the disease, an attempt has been made to coordinate clinical and pathological findings, since they are part of a single process. As is well known, the disease in man follows a rather similar course, and the usually described clinical stages are comparable with those observed in the monkey, except for minor differences in time course of stages. For example, the stage of convalescence is probably of shorter duration, as a rule, in the monkey as compared with man.

DESCRIPTION AND DEFINITION OF GRADES OF PARALYSIS

Limbs were graded as to degree of loss of voluntary muscle power, with flaccidity, hereafter termed weakness or paralysis. Six categories were established arbitrarily, with 5 or "Normal" representing one extreme and 0 representing the other, that of complete flaccid paralysis. It was found possible, with the method of grading used, to obtain excellent agreement upon independent observations by the same or different examiners. The grading for purposes of this report is defined as follows:

5—"Normal"

4—Definite weakness, but capable of using limb effectively in climbing, running, or jumping

3—Uses limb poorly in climbing, running, or jumping

2—Barely able to use limb in climbing or running movements, but able to move limb segments readily against gravity

1—Dangling limb with only feeble movements at skeletal joints

0—Complete flaccid paralysis, or only faint traces of movement

All the gradings were made with regard to maximal possible effort, and with reference to normal function preceding the onset of paralysis. Since individual animals vary greatly in normal strength depending on size and physical condition, any grading more closely spaced could

hardly be comparable from animal to animal, or accurate. The above six gradings roughly correspond to those used by Kristensen and Wulff ('47) for human cases, and follow as closely as possible the percentage grading used by Lenhard and the Kendalls in their human cases ('43). In animals in poor physical condition, even these grades cannot be applied with confidence by an experienced examiner, but in healthy, vigorous animals it was often felt that closer grading could have been used at times. This was especially true in comparing right and left arms or legs, when one member of a pair was slightly weaker than the other. It may be said, in anticipation of the more detailed analysis to follow, that in animals later shown by histopathological study to be definitely poliomyelitic, clinical estimates of muscle power were correlated well with the severity of lesions in anterior horns. In no case was an extremity rated weaker than another in the same animal without a correspondingly greater amount of motoneuron damage in its respective anterior horn. This indicates that a correlation does exist between severity of paralysis and the degree of motoneuron damage, and presently it will be seen how closely correlated these functions are.

DESCRIPTION OF FINDINGS

I Cytopathological Changes in Motor Nerve Cells

The fundamental concept that the primary injury in poliomyelitis occurs in the anterior horn cells, and is the cause of the motor paralysis, was first stated by Charcot (1870). The reasonable objection that his conclusions were gratuitous because based on chronic cases was not soon answered. Acute cases showed inflammatory lesions which secondarily could have affected nerve cells. It was Rissler (1888) who first made an adequate study of acute cases and presented evidence that the primary pathological process occurred in nerve cells, and that inflammatory changes were secondary. The controversy which followed Charcot's publication, however, remained active until experimental evidence was presented indicating that neuron changes could occur independently of inflammatory changes in the preparalytic period (Hurst, '29), and that the inflammatory response did not occur in de-neuronated, but susceptible, nerve centers (Bodian and Howe, '41). That the entire pattern of reaction of non-neuronal elements cannot be accounted for on the basis of a secondary response to neuron changes

alone has been apparent to most modern workers. But, regardless of the complex chain of probably interdependent cellular events which follow the onset of the pathological process, it cannot be doubted now that the neurons are involved in the earliest stages of reaction, and that their injury precedes and is independent of inflammatory cell changes. The presumption at the present time is, therefore, that the regressive changes in motoneurons to be described in this section are the result of direct virus action. Most of the regressive changes may be found when virus is first present in detectable concentration in the anterior horns (Bodian and Cumberland, '47).

Nissl Substance The first microscopic intracellular change seen in motoneurons is the reduction in size and in staining capacity of the Nissl bodies (Hurst, '29, Bodian, '45). This change very largely occurs in diffuse fashion throughout the cytoplasm and is characteristic of most of the abnormal motoneurons seen in the preparalytic period (also Sabin and Ward, '41). The frequency of occurrence of different degrees of severity of such diffuse chromatolysis of Nissl substance is such as to indicate clearly that this chromatolysis is progressive and may lead in some cases to complete disappearance of basophilic cytoplasmic material within a period of hours or less. In one of the earliest preparalytic cases examined (959, Table 3), motoneurons showing mild degrees of diffuse chromatolysis were found in much greater numbers than those showing severe chromatolysis, but this order is reversed in late preparalytic and early paralytic cases. The diffuse chromatolysis of early poliomyelitis is arranged in Plate 1 (figs 1 to 8) to show the probable sequence of progression of the process in cells selected from a single preparalytic case (B12). Plate 2 (figs 1 to 8) shows stages in the continuing progression of degenerative changes which are probably irreversible.

Evidence which will be presented in detail in a later section indicates that the diffuse thinning of Nissl bodies is characteristic of a period of about 5 days after onset of the pathological process. However, in the period between 3 and 5 days after onset of paralysis, a new morphological pattern of Nissl bodies in infected cells also appears. This pattern consists of thickened Nissl bodies adjoining the cell and nuclear membranes, with a central area in the cytoplasm relatively free of Nissl bodies. This appearance we shall refer to hereafter as "cen-

tral chromatolysis" After the first week central chromatolysis is the predominant appearance of Nissl bodies in injured neurons which are not obviously necrotic Such cells were described by Sabin and Ward ('41) as cells with margined Nissl substance, which they observed in cases past the acute stage It will be shown later in this report that central chromatolysis is a manifestation of the recovery process in nerve cells, and that the reconstitution of Nissl bodies near the cell and nuclear membranes is an important phase of the reparative stage in the motoneurons A similar process seems to occur in motoneurons regenerating after axon section (Bodian, '47a)

Mitochondria McCann ('18) appears to have been the first to observe that mitochondria of rhesus motoneurons were apparently unaffected by poliomyelitis virus action even when the Nissl substance had disappeared Hurst ('29) also found apparently normal mitochondria in neurons in which Nissl substance was already completely absent, and even in some cells which were vacuolated Mitochondria were absent in necrotic cells, however Covell ('32) found mitochondria, as well as the Golgi substance, apparently uninvolved in the early stages of poliomyelitis, at a time when chromatolysis, clumping of neutral red granules to one side of the nucleus, and some thickening of neurofibrils was apparent

Our own findings are in essential agreement with those mentioned above Mitochondria as seen in iron haematoxylin preparations were found to be normal in appearance in all stages of injury of motoneurons short of acidophilic necrosis (complete loss of basophilia of cytoplasm) This is shown in Plate 3, in which a severely chromatolytic cell (fig 1) is seen to contain numerous well-stained mitochondria, but a cell in acidophilic necrosis, with severe nuclear changes (fig 2), contains no demonstrable granules In fig 1 the faint outlines of a few remaining Nissl bodies are seen in the cell periphery (above and below)

Since mitochondria are found normal in appearance except in cells showing severe nuclear changes, complete loss of cytoplasmic basophilia, or neuronophagia, it is suggested that dissolution of mitochondria occurs only when cell injury has reached the stage of irreversibility Although it is easily conceivable that chemical changes in mitochondria, and their functional impairment, may precede visible morphological changes, yet it is difficult to imagine that mitochondrial

functions are directly associated with virus activity. Evidence previously presented (Bodian and Cumberland, '47) indicates that virus concentration reaches high levels in the spinal cord at a time when very few cells are at a stage of cell injury accompanied by mitochondrial changes, but when, and only when, many cells show considerable chromatolysis of Nissl substance. One would ordinarily expect some morphological change of mitochondria at the time of greatest virus multiplication, if they were directly associated with this process.

Neurofibrils As has been mentioned, Covell ('32) observed some thickening of neurofibrils in the early stages of involvement of motoneurons, but this was not observed in our protargol-stained material. Well stained and apparently normal neurofibrils were found in all cells, including severely chromatolysed ones, except those showing definite signs of necrosis.

Axons Although the changes in peripheral nerves and nerve roots have long been recognized as resembling those of secondary or Wallerian degeneration, O'Leary, Heinbecker, and Bishop ('32) were the first to study the axonal changes in poliomyelitis in detail, from the functional as well as morphological standpoint, and to review the earlier literature. They clearly appreciated that a correlated study of functional and morphological changes was much more difficult in poliomyelitis than in the case of severed nerves, because in the latter "all axons are in approximately the same stage of alteration at the same time, while in poliomyelitis not all the cells are affected to a like degree at once." Nevertheless they established conclusively certain important facts. They showed first that motor paralysis precedes any noticeable morphological or functional changes in the axons or muscles, and that therefore the onset of paralysis is due to loss of conduction through the diseased motoneurons in the spinal cord. Moreover, the axon lesions when they appear are similar to those occurring after nerve section and occur more or less simultaneously along the length of the axon. Although they found increased irritability of fibers in affected trunks during the preparalytic and early paralytic period, they thought that the period between loss of synaptic conduction and degenerative changes in motor roots and peripheral nerves was significantly longer than that which separates nerve section and subsequent changes in peripheral stumps. This is readily explained on the basis that moto-

neurons so much damaged that they no longer conduct can still exert an adequate trophic influence on their axons

The time course of axon changes described by O'Leary and his colleagues agrees with that which we have observed, except our material clearly shows that definite degenerative changes in ventral root fibers may occur as early as the third day of paralysis, whereas O'Leary, et al found no changes before $4\frac{1}{2}$ days. Our material consisted of reduced silver preparations of spinal cord with roots attached, permitting easy identification of roots issuing from regions with completely destroyed motoneurons. It is in such roots that one finds numerous degenerating axons on the third day of paralysis. Since some of the nerve cells giving origin to these fibers are probably destroyed on the day preceding onset of paralysis, the interval between cell destruction and visible axon deterioration is no longer than four days, and in some cases probably less. This agrees so closely with the time course of axon degeneration after nerve section (two to four days—Heinbecker, et al, '32), that the conclusion is inescapable that the degeneration of ventral root axons in poliomyelitis is a typical Wallerian degeneration. O'Leary, et al ('32) point out that actively degenerating fibers may be found as late as 10 days, and later, following onset of paralysis, and suggest that this indicates the delayed death of cells of origin as late as 10 days after onset of paralysis. That this is not common will be shown in Part II, since very few cells show necrotic changes so late after onset of paralysis. A more probable explanation of their findings is that active degeneration of axons continues for several days after some axons have begun to deteriorate. It is interesting that Blanton ('17) found degenerative changes in the peripheral nerves of a human patient dying 36 hours after onset of paralysis. Allowing at least 24 hours for motoneuron destruction in the pre-paralytic period, one concludes that at least $2\frac{1}{2}$ days could have elapsed between cell death and axon deterioration. Although Blanton thought the changes he described were too early to be due to ventral horn destruction, his findings are not contrary to the time course of typical secondary or Wallerian degeneration.

The delay in onset of axon degeneration after cell body destruction in poliomyelitis is beautifully illustrated in reduced silver preparations of the spinal cord during the first few days of the disease. In some

sections numerous motoneurons were seen, consisting only of a rounded, pale-staining mass at the site of the axon hillock, and in continuity with an axon of normal appearance. In some cases these axons were traced out into the ventral roots in the same section and were seen to be of normal appearance throughout. This picture of cell body remnant with intact axon was first illustrated, as far as could be determined, by Strauss ('10), in material from a patient who died the day after onset of paralysis.

In summary, our preparations of monkeys with abrupt onset of severe paralysis show clearly that ventral root axons of motoneurons destroyed on the first day of paralysis, and probably many on the preceding day, are morphologically intact until the third day after onset of paralysis, when degenerative changes occur all along the axon length. On the 1st and 2nd day of paralysis axons appear to degenerate centrifugally from the cell body for a short distance. It is interesting that even when inflammatory changes are most intense, including neuronophagia, no inflammatory cells of any kind are found anywhere along the axons of cells undergoing active phagocytosis. The conclusion is inescapable from this fact, and from the time course of axonal degeneration, that the primary focus of virus activity is in the nerve cell body, and that, when the cell body is destroyed, the axon undergoes typical Wallerian degeneration. Plate 4 shows the sequence of changes in anterior root fibers of limbs severely paralysed on the first day of paralysis. Figure 1 shows a normal anterior root. Figure 2 shows a degenerating root fiber in the white matter of the anterior column in an animal killed on the 3rd day after onset of paralysis. Figure 3 shows the almost universal degeneration of fibers in the anterior root of an animal killed on the 4th day. Figure 4 shows the complete absence of axons in the root of an animal killed 57 days after complete paralysis of the corresponding limb.

Nuclei Changes in nuclei of motoneurons in acute experimental poliomyelitis have been described by Covell ('30), by Hurst ('31), and by Sabin and Ward ('41), especially with reference to the occurrence of acidophilic "inclusion bodies." Among the important findings they reported, and which our material confirms, is the fact that acidophilic inclusions, either single or multiple, are seen only in cells already showing severe cytoplasmic changes. In our experience this

holds true not only for the formation of inclusion bodies but also for the occurrence of morphological nuclear changes in general. Strauss ('10) and Blanton ('17) found the same to be true in human material. Covell found that the acidophilic inclusions gave a negative Feulgen reaction, indicating the absence of thymonucleic acid, and also reported that the inclusion bodies were most numerous soon after the onset of paralysis and diminished in numbers during the 1st to the 3rd weeks. Our findings are similar but we have observed considerable variation in the frequency of occurrence of intranuclear inclusions in different monkeys in the acute stage of the disease.

Our material indicates that nuclear changes begin after cytoplasmic changes occur, since cells showing early chromatolysis exhibit no signs of nuclear injury. Clumping of oxychromatin and the formation of discrete acidophilic inclusions occurs in cells showing fairly severe chromatolysis. In parallel with continuing cytoplasmic degenerative changes, nuclei show an apparently orderly progression of changes, as shown in Plate 1, figs 5-8. Most commonly one notes progressive shrinkage of the nucleus and an increase in discrete chromatin masses, with progressive increase of cytoplasmic chromatolysis. When severe nuclear shrinkage is seen (Plate 1, fig 8, Plate 2, figs 1 and 2) the nuclear membrane is usually distorted or no longer visible, and the nucleus itself becomes intensely basophilic. The diffuse basophilia which is seen in some nuclei at this stage is usually paralleled by a similar increase of diffuse basophilia in the cytoplasm. Such cells appear to be most prone to undergo neuronophagia (Plate 2, fig 3), and even in earlier stages seem to especially attract leucocytic and phagocytic elements. By the time phagocytosis due to granulocytes and macrophages is well under way, however, the cytoplasm has generally lost all basophilic staining (Plate 1, fig 3).

In cells which undergo rapid lysis, or show severe vacuolation (Plate 2, figs 6-8), the nuclei do not show the generalized basophilia noted above, and this again parallels the lack of basophilia in the cytoplasm. Such cells are not as commonly surrounded by large numbers of leucocytes and macrophages as are those showing terminal basophilia. Very often in cells undergoing lysis, the nucleus may be quite shrunken, but with basophilic granules only encircling the periphery, and with a nucleolus greatly reduced in size (Plate 2, fig 6).

In a previous report (Bodian, '45) evidence was presented showing that the nuclei of any multinucleated motoneuron underwent parallel degenerative changes, such as shrinkage, distortion of nuclear membranes, and formation of acidophilic inclusions. The remarkable similarity in morphology of the nuclei of any multinucleated cell, in all stages of degeneration due to virus action, suggested that nuclear changes in poliomyelitis reflect the deterioration of cell functions in general rather than a direct action of virus on the nucleus.

The nuclei of persistent cells which are apparently irreversibly damaged may show various morphological appearances too diverse to suggest an orderly sequence of changes. In such cells showing evidence of retarded death and absorption the nucleus is often intensely basophilic (Plate 5, figs 2-4), may be swollen or shrunken, or may appear to be relatively intact, as if resisting degeneration more than the severely injured cytoplasm (Plate 5, figs 1 and 5). In some of these cells the nucleus appears to be on the verge of being extruded, although relatively normal in appearance (Plate 5, fig 6, Plate 10, fig 1). The cytoplasm in persistent severely damaged cells is generally highly eosinophilic or hyalin in appearance. At times such cells exhibit a ring of peripheral Nissl substance of abnormal appearance, quite unlike that seen in most cells in the stage of central chromatolysis.

In the subacute stage and later recovery periods, the nuclei of cells with normal cytoplasmic structure are generally normal in appearance as well. Occasionally, however, large persistent acidophilic inclusion bodies are seen in cells of otherwise quite normal appearance (Plate 9, fig 4). Since in the acute stage such inclusions are seen only in severely chromatolysed cells, their presence later in cells otherwise normal is evidence of the power of recovery of cells damaged by virus action. Such inclusions have been seen in otherwise normal cells as late as 49 days after onset of paralysis. Rarely one sees in otherwise normal cells an unusual nuclear "inclusion" consisting of the nucleolus surrounded by a closely packed aggregate of basophilic material (Plate 9, figs 5 and 6). This appearance is also occasionally seen in severely damaged cells (Plate 10, fig 2). This bizarre intranuclear formation in otherwise normal cells has not been seen in formative stages, so that no interpretation of their development seems feasible at present.

Criteria of Necrobiosis in Motoneurons It is evident from the pre-

ceding paragraphs that certain extreme cytopathological changes were associated with the processes of necrobiosis or irreversibility of injury following poliomyelitic infections. Among the irreversibly injured cells are, of course, those destroyed and completely absorbed, and those which persist for variable periods of time before being absorbed, and designated hereafter as "necrotic". The process of cell destruction and complete absorption in poliomyelitis must occur at times in the space of a few hours, since in our earliest preparalytic cases one already finds cells in this category whose only mark is either a vacant space in the section ("falling out"), or a cluster of phagocytic cells filling in the spaces formerly occupied by the neurons. Many nerve cells, however, die more slowly, presumably after passing through the usual stages of chromatolysis. Unlike the early stages of degeneration no single orderly sequence of morphological stages characterizes the processes of deterioration from the time of cell "death" to the time of complete disappearance of the cell. Cells which we have designated as necrotic may show severe chromophilia or complete loss of stainability ("ghost cells"), vacuolation and lysis without neuronophagia or signs of neuronophagia without lysis, severe nuclear change including pyknosis or extrusion of a relatively intact nucleus. The disorderly variation of morphological appearance after complete loss of Nissl substance suggests first that such complete loss is incompatible with recovery of the cell, and second, that subsequent cellular processes are quite out of control and may lead to several alternative morphological sequences leading to complete cell destruction. It is interesting that Horanyi-Hechst ('35) has described quite similar terminal stages of motoneuron degeneration in human cases.

In *summary*, the following cytological changes appear to be associated with irreversible neuron injury, and have been considered as indicators of necrosis

- 1 Complete loss of cytoplasmic basophilia
- 2 Disappearance of mitochondria
- 3 Breakdown of neurofibrils
- 4 Severe nuclear changes, other than the formation of "inclusions". This includes severe nuclear pyknosis, complete loss of nuclear basophilia, rupture of nuclear membrane, or nuclear extrusion
- 5 Cytoplasmic vacuolation
- 6 Glassy appearance of cytoplasm, with some Nissl substance still remaining in the periphery
- 7 Severe cellular basophilia, usually associated with beginning neuronophagia
- 8 Neuronophagia

II The Sequence of Pathological Changes in the Anterior Horn, with a Statistical Study of Injury and Recovery Stages in Motor Nerve Cell Populations

It is possible to examine individual nerve cells in poliomyelitis in various stages and to draw certain inferences regarding the sequence of events in their injury and recovery stages. Heretofore, this has been done largely with respect to the injury stages by workers like Covell ('32), Hurst ('29), and Sabin and Ward ('41). Many of such inferences, although reasonable, are impossible to prove without quantitative data regarding the cell population as a whole, and moreover cannot be related to the entire disease picture without a statistical approach. Such an approach should be capable of revealing important data impossible to obtain by the study of individual cells. The pathogenesis of paralysis, for example, bears no relation to the fate of any individual cells, since the cells in the nerve cell population supplying the arm of a rhesus monkey number about 14,000 and at any particular time in the acute stage may be in widely varying stages of pathological change. The paralysis can only be related to the status of the cell population supplying the muscle or groups of muscles under consideration. Similarly, at the present time, the concentration of virus in infected tissue can be related only to a cell population in the tissue being studied.

It is the purpose of the following pages to present first a survey of the principal changes occurring both in the motoneuron population and in the populations of reacting mesodermal glial cells during sequential clinical stages of the disease. With this survey in mind, the statistical analysis of changes in the motoneuron population will then be more readily understood.

Pathological Characteristics of Successive Clinical Stages

The preparalytic period

The most informative stage in the pathological process is the earliest in which visible changes are apparent. This stage precedes paralysis by about 1 to 3 days, and gives important information about the sequence of early changes in neurons as well as in the inflammatory response. The important findings may be summarized as follows, before we proceed to the details.

1 The most common visible change in nerve cells in the earliest stage is a diffuse thinning of Nissl bodies throughout the cell body, often most conspicuous around the nucleus. Various degrees of this may be seen, but it is only in the late preparalytic stage that complete dissolution of Nissl bodies is found in large numbers of cells.

2 Nuclear changes are, as a rule, not apparent until severe cytoplasmic chromatolysis has occurred. The earliest nuclear change is usually the formation of acidophilic inclusion bodies.

3 Changes in mitochondria are not apparent in cells showing mild or even severe chromatolysis (also McCann, '18, Hurst, '29) but very few mitochondria remain when presumably irreversible changes have occurred in the cell ("acidophilic necrosis", nuclear dissolution or pyknosis, cell vacuolation, neuronophagia).

4 Neurofibrillar changes are not apparent until severe cell changes have occurred (also Hurst, '29).

5 The entire sequence of neuron changes from early injury to cell necrosis is present in the earliest pathological focus in our series, although frankly necrotic cells are few in number. Since recovery changes in nerve cells at this time are hardly to be expected, all changes may be assumed to be progressive and have been arranged in sequence, according to severity, in Plate 1 (figs 1-8).

6 Changes in nerve cells of mild or severe degree in the preparalytic period are entirely independent of inflammatory changes, as shown by Hurst ('29), and may occur in regions of ventral horn gray matter where no inflammatory response has occurred. Nevertheless, leucocytes may be present in considerable numbers in other regions where nerve cell changes are so slight that they are not clearly outside of the range of normal.

Monkeys 959 and A935 were most instructive in revealing the earliest inflammatory and neuronal changes in our series. Rhesus 959 was inoculated intracerebrally with MV virus 5 days before he was killed, at which time the only symptom was that of fever, present for 24 hours. No lesions were present in serial sections of the lumbar cord. The cervical cord contained only two pathological foci, one restricted to about 100 15μ sections within the 6th cervical segment, and the other restricted to 32 sections within the 7th cervical segment. The focus in C6 contained no interstitial infiltration, but only very light

perivascular infiltration in the dorsal parts of the anterior horns and in the intermediate gray horns, especially around the branches of vessels in the ventral fissure. The infiltrating cells were largely granulocytes, with a sprinkling of mononuclear cells. Within this region of the cord no definite changes in nerve cells were visible except chromatolytic changes which were so slight that they could have been within the range of normal.

The focus in C7 was more advanced than that in C6 and was remarkably restricted to the left anterior and intermediate gray horns, both with respect to neuronal and to inflammatory changes. The only vessel outside of this territory showing perivascular infiltration was the artery of the ventral fissure, and only the left branches of this vessel were "cuffed". Within this focus the perivascular infiltrations contained more mononuclear cells and relatively fewer granulocytes than in the earlier focus in C6. Perivascular leucocytes were present around vessels of all sizes from capillaries to the vessels of the ventral fissure. At the periphery of this focus there were still no diffuse or focal extravascular infiltrations, although some neurons showed diffuse cytoplasmic chromatolysis of a definite degree. These neurons showed no nuclear changes as yet and no leucocytes or excess of glia cells were to be seen near them. In the center of the focus, granulocytes and some lymphocytes were seen in the parenchyma in small numbers as well as in perivascular spaces. Granulocytes could be seen surrounding neurons of normal appearance, or around cells in any stage of chromatolysis or degeneration. They were numerous in the vacuoles or "bays" of hyperchromatic necrotic neurons, but were rarely seen near "ghost" neurons or severely autolysed ones. It is important to note that lightly or severely damaged neurons may or may not be surrounded by infiltrative cells. One may therefore conclude that, although breakdown products associated with the earliest neuronal changes can attract migrating leucocytes from the vessels, not all damaged neurons exert this influence.

The cytological changes in the neurons of this focus were of great interest. Within the 32 15μ sections in the segment of cord which contained the focus it was found by actual count of all motoneurons that there were a total of 442 in the lateral column of the right anterior horn, and 451 in the corresponding region on the left side. All neurons on the

right side were normal in appearance and no infiltrative changes were present. On the left side 52% of the neurons were normal in appearance, 28% showed mild degrees of diffuse cytoplasmic chromatolysis, without nuclear changes, 16% showed severe cytoplasmic chromatolysis, and such cells often contained nuclear inclusion bodies, and 4% of the neurons showed necrotic changes. Only one cell was in the process of neuronophagia. In all other preparalytic cases, a similar distribution of pathological cell types was found, with however more cells actually destroyed or necrotic (Table 3). Thus, on the basis of frequency of occurrence, one can safely reconstruct the sequence of neuronal changes from the earliest visible ones to the stages of cell breakdown, as shown in Plates I and II.

It is interesting that in the preparalytic period, among six animals examined in detail, it was exceedingly rare to find cells showing central chromatolysis, although this pattern of Nissl body distribution is quite common in the subacute stage, as will be seen. Moreover, in no regressive stage in the preparalytic period does one find the nuclear eccentricity characteristic of the chromatolysis which follows axon amputation.

In later stages of the preparalytic period, polyblasts may be present in large numbers as well as the numerous leucocytes. Granulocytes appear to be more numerous in the lumbar than in the cervical cord as a rule, as also noted by Hurst ('29), by Pette, et al ('32) and by Covell ('32). This was also true in Blanton's earliest human case of 36 hours' duration ('17). In some individuals they may be present in enormous numbers in the gray matter, and in such cases appear to migrate into the surrounding white matter in large numbers.

Another preparalytic case (A935) is especially instructive in revealing very early pathological changes, and in emphasizing two important aspects of pathogenesis. In this case, which at some levels of the cord shows characteristic and fully-developed neuronal and inflammatory lesions (Plate 6, figs 1 and 2), one finds at adjacent levels sections which contain only a single motoneuron of abnormal character (Plate 6, figs 3, 4 and 5). Such isolated neurons showed the severe diffuse chromatolysis typical of early poliomyelitis and occurred in several sections. Of considerable interest is the fact that no infiltrative cells were present in the anterior horn gray in the region surrounding such

neurons, although early perivascular infiltration was present surrounding the main branches of the anterior spinal vessels (Plate 6, fig 2) Such findings strikingly confirm Blanton's ('17) in human cases and Hurst's ('29) in monkeys, in demonstrating that neuronal chromatolysis, even of severe degree, may occur prior to and not as a result of inflammatory changes It is interesting that Pette, Demme and Kornyei ('32) also found early neuron changes in the absence of cellular infiltration, but were not sure that such changes were not artifacts of histological preparation In our material, the isolated chromatolytic cells were found in any position of the anterior horn cross-section, whether dorsal, ventral, medial, or lateral There was no suggestion of a progression of lesions from the dorsomedial position of the horn lateralwards, as postulated by Elliott ('45) At least in the early stage of the disease, the spread of virus in the gray matter does not occur by cell-body to cell-body extension, but probably along axon connections in which cytopathological changes cannot be observed The question is raised, in this connection, whether the often postulated spread of virus liberated from destroyed cell-bodies to adjacent cell-bodies occurs at all, since, as will be shown below, extensive dissemination of neuron lesions may occur before widespread nerve cell destruction Moreover, the highest virus titers may be achieved in the preparalytic period before extensive cell destruction occurs, and presumably as a result of virus multiplication in many cells (Bodian and Cumberland, '47) In cases with little paralysis, inoculated with "mild" strains, extensive motoneuron chromatolysis occurs and high titers are obtainable, with only a small proportion of destroyed nerve cells

The first day of paralysis

In animals in which the onset of paralysis is abrupt, histopathological changes in the spinal cord resemble closely those seen on the day preceding expected paralysis, but are generally more widespread and more severe Although the predominant cytopathological stage in motoneurons is that of diffuse chromatolysis, numerous cells in various stages of necrosis and neuronophagia can usually be seen Such cells are most common at this time as compared with preceding or following days Advanced stages of chromatolysis of Nissl substance, including

complete disappearance of the latter, predominate, with some cells showing cytoplasmic vacuolation or autolysis. Nuclear changes are rarely severe except in cells showing severe chromatolysis, and in such cells intranuclear acidophilic inclusions are common. Acidophilic inclusions generally occur in cells with otherwise relatively normal nuclei whereas basophilic intranuclear "inclusions" are found at times in cells which have either shrunken or highly vacuolar nuclei.

Neurons about to undergo neuronophagia are common at this stage, and are generally highly basophilic. In later stages of active neuronophagia neurons are strongly eosinophilic, suggesting perhaps that the neurophages produce enzymes, possibly nucleases, which remove the basophilia. Many neurons are destroyed, become autolysed, and are absorbed without any evidence of participation by neurophages, and both removal by autolysis and by neuronophagia may be so rapid that even on the first day of paralysis the location of many motoneurons may be marked only by a vacant space ("falling out"), or by a compact cluster of neurophages.

The inflammatory process on the first day of paralysis is in general more severe than on the preceding day, with a tendency, however, for reduction in numbers of granulocytes. The presence of much cellular debris, and apparently nuclear fragments, in some foci may mark the site of heavy infiltration with such cells, and suggests that their rapid disappearance is due to destruction *in situ*. During the period of early and increasing paralysis the lymphocytic cells are predominant in numbers, although macrophages are also quite numerous.

Second and third days

The outstanding feature of the histopathological picture at this period is the scarcity of normal-appearing neurons, even in cases with mild paralysis. Not a single case examined at this period showed more normal cells than 31% of the expected number. This indicates the wide dissemination of virus in the spinal cord of paralytic cases, and also supports evidence from other sources, to be considered later, that many cells are invaded by virus and later recover. Residual neurons are predominantly in late stages of diffuse chromatolysis, although, for the first time, cells showing central chromatolysis may make their appearance in appreciable numbers.

At this period most of the destroyed neurons are completely absorbed, with only focal accumulations of neurophages to mark the spot. Early stages of neuronophagia are also seen, but are not usually as numerous as on the first day of paralysis. In regions of extensive cell loss, even the foci of neuronophagia may have cleared up, so rapid is the sequence of changes, and large areas of anterior horn, devoid of nerve cells, may be characterized by a diffuse increase of mononuclear cells.

The principal cells in the inflammatory response, at this time and in most cases, are the polyblasts of Maximow, as shown by Wickman ('13). These cells, with rod-shaped or irregular nuclei, have also been identified as microglia by various observers. Other cells of the lymphocytic series may also be very numerous, especially in the perivascular accumulations of cells. Normal-appearing granulocytes are uncommon, and their disappearance as such is marked by the occurrence of much basophilic nuclear debris which probably represents their remains.

Silver preparations at this stage show the earliest phases of axon degeneration in ventral root fibers, occurring only in cases killed at least $2\frac{1}{2}$ days following the onset of paralysis. These changes have been described in the first section of this report, and require no further comment.

Four to six days

This period is remarkable from the morphological as well as from the functional point of view. The cases, like those in the preceding period, show a very low percentage of normal neurons, even in cases with surprisingly little functional loss, and with cessation of increase of paralysis. Since very little, if any, active neuronophagia is in evidence, and since necrotic stages of any sort are rare, it is likely that surviving cells would have recovered even though many are severely chromatolytic.

This period is also interesting in showing a reversal of the striking tendency of damaged neurons in the preceding periods to show diffuse chromatolysis in the cytoplasm. Now many cells show aggregation of Nissl substance near the cell and nuclear membranes. Cells showing this type of "central chromatolysis" (Plate 7, figs 4-6) are mixed with those showing the diffuse type (Plate 7, figs 1 and 2), and many

transitional stages are also seen (Plate 7, fig 3) Occasionally in the same individual one finds cells with diffuse chromatolysis predominating in one spinal cord segment and those with central chromatolysis predominating in a neighboring segment Eccentricity of nuclear position appears in some cells showing "central" chromatolysis

The axons of motoneurons reveal interesting changes at this stage In segments of spinal cord in which extensive destruction of motoneurons has occurred, many axons in the ventral roots show severe degenerative changes, and, in some cases, no intact axons are visible (Plate 4, fig 3) In segments where many neurons remain, most axons in the roots are normal in appearance, *even when most neurons show severe chromatolytic changes* This finding confirms other suggestions, previously mentioned, that axon degeneration is a consequence only of actual cell body destruction

Seven to twelve days

The principal feature of neuron morphology in this period is the predominance of "central" chromatolysis in remaining cells other than normal-appearing ones (Plate 8, figs 1-5) Cells showing diffuse chromatolysis or necrotic changes, as previously defined, are few in numbers as a rule The one exception to this rule was found in case B23, in which many cells in severe diffuse chromatolysis were found This suggests a possible association with continuing virus activity, since in exceptional cases this may be found as late as the third week after onset of the disease (Brodie, '33, Bodian and Cumberland, '47)

Several features of the pathological picture in this period give evidence that the recovery process is well under way A greater proportion of normal or almost normal neurons is found than in the preceding period In some cases numerous cells are seen with normal or almost normal Nissl body pattern, in which, however, large inclusion bodies are found in the nucleus, indicating previous cytoplasmic changes of a fairly severe degree (Plate 8, fig 6, b) Although the inclusions are usually acidophilic, many at this stage in cells of otherwise normal appearance are larger than usual and seem to consist of an aggregate of basophilic particles which surrounds the nucleolus, converting it into a core for this bizarre formation Such nuclear "inclusions" are generally surrounded by a shrinkage space, and are observed as late as

35 days after onset of paralysis (Plate 9, figs 5 and 6, a) Although the cytoplasm in such cells is generally normal or almost so, it is problematical whether cells with such marked nuclear change can continue to recover, since in a few cases cells with nuclear inclusions of this type were seen in a condition of obvious necrosis (Plate 10, fig 2) Finally, in this period there is a tendency for disappearance of lymphocytes which were previously scattered throughout the parenchyma in company with polyblasts or macrophages At the same time the number of lymphocytes in the perivascular infiltrations increases considerably, so that "cuffing" is more prominent than before This increase is in part due to formation of lymphocytes *in situ*, as evidenced by numerous mitotic figures, but may also be aided by accretion from lymphocytes free in the tissue The disappearance of the lymphocytes free in the tissue, however, is probably more often due to transformation into polyblasts, as described by Maximow ('27) Some lymphocytes appear to undergo phagocytosis by macrophages, but this is not often observed (Plate 10, fig 2, m) At any rate, during the second week the population of "replacement" cells in the parenchyma is predominantly polyblasts, and variations of these such as "gitter" cells and "rod" cells are numerous Although the polymorphonuclear leucocytes have a short period of prevalence as infiltrating elements, namely the first day or two of the disease, and cells of the lymphocyte group a somewhat longer period, the first week of the disease, the histiocytic cells or macrophages are the most prevalent after the first week and predominate among the non-neuronal cellular elements for several months

The third week

During the third week after onset of paralysis, less than 10% of all neurons are still abnormal in appearance, so rapid is the recovery process Most of the nerve cells, by this time, are either normal in appearance or destroyed and removed without remaining traces A few irreversibly damaged cells of the type shown in Plate 5, figs 1-6, may be seen in some cases as late as this period, but such persistent "necrotic" cells are negligible in numbers as compared with those quickly destroyed and removed during the first few days of the disease The predominant pathological stage is that of "central" chromatolysis, with evidence of recovery of Nissl substance (Plate 9, figs 1 and 2) Intracellular inclusions may be present (Plate 9, fig 2, b)

The increased size of the perivascular infiltration noted in the previous period, is even more striking in some cases during the third week, when some of the larger 'cuffed' vessels attain maximum proportions. Around other vessels, especially the smaller ones, the lymphocytes tend to disappear, and are replaced by smaller shaped histiocytes. This process is seen as early as the second week after onset of paralysis (Plate 10, fig. 2), but is more common during a later period. It is interesting that occasionally one finds at the same vessel both of heavy lymphocytic hyaline zones and also smaller cells, the latter greatly predominate in the thinned adventitial layer. In the perenchyma itself in cases during the third week, a few small vessels are found, with a small spread histiocyte infiltration.

Third, fourth and fifth weeks

The cytopathological picture in the case of the second group is that in the preceding one. However, contrast is with the previously normal appearance and which might be considered as the normal state found. A very small proportion of cells, both in the nerve and in the muscle, are observed (Plate 9, fig. 3 to 6), and after a few days the cells are essentially normal in appearance. Large pale bodies, resembling foamy bodies were found in a few cells in other sections, especially in the muscle (Plate 9, fig. 4, b), as late as 10 days after onset.

The persisting lymphocytic perivascular infiltration in some instances assume the proportions of small lymphocytic infiltrates in the period (Plate 11, fig. 1 and 2). Other regions of the cord, however, individual however, may be nearly free of lymphocytes at this time and exhibit histiocyte as infiltrating cells, but tend to be very few. In some cases the heavily 'cuffed' vessels show hyaline zones and cells in the center of the mass of lymphocytes and mitotic figures which give clear evidence of active lymphocytopenia (Plate 11, fig. 1).

Conclusions three

The morphological recovery of over 96% of surviving neurons is attained at the end of the first month of the disease. During the next few weeks, as indicated above, the residual chromatolytic cells are negligible in number, and moreover the number of lymphocytes free in the tissues is reduced to very small proportions. It is interesting that the essential disappearance of leucocytic infiltration, which

marks the close of the active inflammatory process, should be roughly coincident with the recovery of most remaining neurons. Both processes take place during the subacute period, following which the further stages of recovery appear to take place at a slower rate. Occasional chromatolytic cells may still be found several months after onset of the disease, for example. Similarly, heavily cuffed vessels may persist for at least a year, although such cuffing is gradually reduced markedly after the first 6 months of the disease. During the late convalescent period histiocytes also are greatly reduced, and affected regions of anterior horn are characterized by a variable degree of gliosis, which marks the terminal stage of the non-neuronal reparative process.

Summary of Quantitative Data

Dynamic Aspects of the Infection and Recovery of the Motoneuron Population

When changes in motoneurons during a series of stages in the acute period are considered in a qualitative way, the variety of morphological appearances is such as to render difficult a clear appreciation of the significant phases of a continuous process. In their very thorough study of histopathological changes in the acute period, for example, Pette, Demme, and Kornyei ('32) were more impressed with the variety of neuron changes than with a possible orderly progression of changes. This problem is easily dissipated when the disease in the spinal cord is considered as an "epidemic" occurrence in the nerve cell population. With this point of view in mind, the *prevalence* of each stage in the regression and recovery of nerve cells can be treated in a quantitative way throughout the course of the disease. Since in each animal the motoneuron population is studied at a single point of time in the disease, and since considerable variability in the severity of the disease occurs from animal to animal, it was decided to select at each principal stage a group of about five animals with approximately the same average severity of paralysis. This represents a group of 20 limbs, supplied by motoneurons of which a total sample of about 10,000 was considered in the quantitative study. The prevalence of each morphological stage was tabulated for the motoneuron population supplying each extremity, and the average prevalence for all extremities in

all animals of a given group then obtained (Tables 3 to 10, Charts 2 and 3) The averages allow a clear understanding of the time course of pathological changes, as shown in the charts, whereas any given animal may represent an extreme case of variation from the normal which may be difficult to assess in relation to the common sequence of events

For the purposes of an epidemiological study it is fortunate that the nerve cell population in an infected animal is absolutely stable with respect to factors such as migration and multiplication The number of cells can be presumed to be constant in number and location, except for decrease by deaths due to the disease, since mature neurons do not multiply or migrate It is necessary only to define the precise cell population involved, and to treat the frequency of occurrence of each stage in the regression and recovery of nerve cells in a quantitative way throughout the course of the disease

Determination of the distribution of the normal motoneuron population
In order to assess the damage to motoneurons due to poliomyelitic infection it was necessary to identify the motoneuron population in normal animals and to establish the pattern of distribution of the neurons which principally innervate each limb Since there is some admixture of such neurons with non-motor internuncial neurons in the anterior horns, the motoneurons were identified by means of the method of retrograde chromatolysis In four monkeys the entire left brachial plexus was sectioned in the subclavian region, and the left lumbosacral roots were sectioned in the intervertebral canals In three additional monkeys, the brachial plexus alone was sectioned After periods of from 7 to 30 days, during which time the cells of origin show clear-cut chromatolysis, these animals were prepared and examined in a manner similar to that described above

After interruption of the limb plexuses it was found that only about 1% of the chromatolytic cells were in positions outside the lateral and central cell columns of the anterior horns The apparently displaced chromatolytic cells were entirely in the medial cell columns and were probably the result of operative injury to the back muscles Since the area of sections containing the chromatolytic cells was therefore essentially the area containing the lateral and central cell columns, it could readily be identified in normal or pathological material The limits of

this area dorsally and dorsomedially can be defined for all practical purposes as the limits of the very large cells (cell bodies over 50μ) In the lumbar region this area is particularly clearly defined, as shown in Plate 12

The chromatolytic preparations also served to differentiate the motoneuron population from internuncial cells in the same area and to show that the latter introduced but a small error in studying poliomyelitis preparations, providing that only cells over 25μ in diameter were considered It was found, for example, that 80 to 95% of cells over 25μ in diameter showed chromatolysis in the lateral and central cell columns after root or plexus section Only about 30% of cells smaller than 25μ showed chromatolysis, and this is probably a high figure since some small cells show apparent chromatolysis in quite normal animals Since such small cells represent only about 20% of the total cell population in the region considered, an error in estimating motor cells of at most 6% is introduced by neglecting small cells These relationships are shown graphically in Chart 1

Chart 1 also shows the variation in both normal and chromatolytic cells in various segments of the spinal cord enlargements As can be seen by examining the curve of mean values, there is a definite trend towards greater concentration of large motoneurons in the two cervical segments C7 and C8, and in the two lumbar segments, L6 and L7 Consequently an average figure for the number of motoneurons on one side in a single 15 micra section was derived for each segment from counts of normal preparations This is shown in Chart 1 for large and small neurons separately, and the total number of cells per section shown in comparison with the number showing chromatolysis after plexus section

In order to determine the number of motoneurons destroyed in poliomyelitic cases, the number obtained by counting the large cells (over 25μ) in a sample of 10 to 15 sections per spinal segment was subtracted from the number which one would expect to find if the cord were normal The "expected number" was obtained by multiplying the number of sections examined in each segment by the average number of large cells per side per section previously determined for the segment in question The sum of the totals of individual segments was then the "expected number" of large cells which should have been present on one side in the sections taken as a sample of the cord enlargement

The "expected number" is thus close to being the average number of motoneurons present in a sample of 40 to 60 sections through a limb enlargement, and supplying a single limb. It is subject to the errors of including about 10% of large cells which may not be motoneurons, of

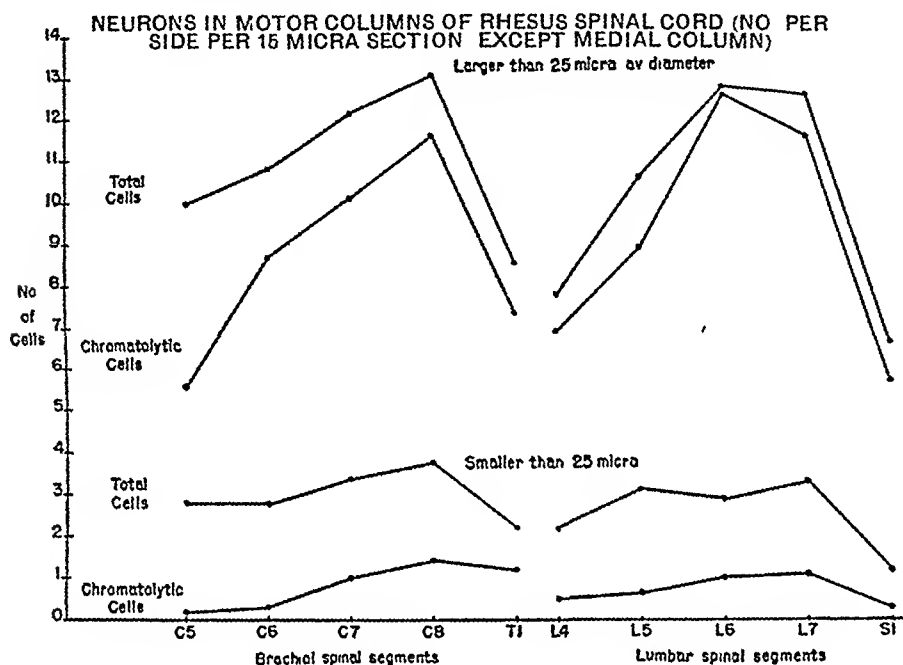


CHART 1 FREQUENCY DISTRIBUTION OF ANTERIOR HORN CELLS IN LIMB SEGMENTS OF RHESUS SPINAL CORD

Cell counts were made in area of cord containing neurons over 50μ in diameter, and showing chromatolytic cells after plexus or root section. Chromatolytic cells in brachial region are the consequence of brachial plexus section, whereas those in lumbar segments are due to section of third to seventh lumbar roots. Each point on the curves represents the combined means of values from 7 or 8 monkeys, except for the small cells, which are obtained from 2 monkeys. In each monkey the average value for cells per section in each spinal segment was obtained by counting the cells in a sample consisting of 10 to 15 sections. Only cells containing nucleoli were counted.

excluding about 6% of small cells which may be motoneurons, and of an individual variation from case to case of about 10%, when the whole region is considered. Errors of this magnitude are not serious in comparing neuron destruction with loss of motor function, since the clinical

estimate of muscle power of a limb is less accurate than the estimate of the number of cells in the corresponding motoneuron population

As a further and final check on the accuracy of determination of the normal control motoneuron population, a comparison was made between the total "motoneuron" count on one side of the brachial (C6 to T1) and lumbar enlargements (L4 to L7) and the counts of nerve fibers in corresponding ventral roots in rhesus monkeys made by Larsson ('38). In reduced silver preparations Larsson found a total of 15,795 fibers in the ventral roots of C6 to T1 inclusive, and 16,347 fibers in the ventral roots of L4 to L7 inclusive. On the basis of our counts of all chromatolytic cells, large and small, in the lateral and central cell columns of corresponding cord segments in our material, an estimated number of 12,000 for the brachial and of 12,600 for the lumbar enlargements was obtained. The latter figures, of course, did not include cells in the medial column which contribute axons to the ventral roots, and which therefore are represented in Larsson's figures. Although Larsson's counts were based on a single rhesus monkey, the agreement between our figures, after discounting the discrepancy based on our omission of cells in the medial column, suggests a reasonably good correspondence between ventral root fibers and cells which we have defined as "motoneurons".

Changing proportions of normal, abnormal, and destroyed cells in the total motoneuron population. A general survey of the course of the disease in the motoneuron population can be obtained by an analysis of the changing proportions of three principal groups of cells in the total population, the normal cells, the abnormal ones, and those totally destroyed. The total population is estimated in the manner previously described and called the *expected number* of nerve cells. The destroyed cells are estimated by subtracting the combined groups of surviving normal and abnormal cells from the expected number.

In Tables 3 to 10 these data are given for each case in the columns headed "Per Cent of Expected Number", and the mean values for all cases in each group are plotted in Chart 2. Although, as might be expected, the variation in severity of maximal paralysis is associated with variation in proportion of cell groups in individual cases from the same period, the mean values clearly reveal the trend of events in the populations. The same trends are reflected to a lesser degree in the maximum and minimum values for each, shown in Tables 3 to 10.

TABLE 3
Preparalytic Period

Analysis of types of neuron damage in samples of motoneuron population supplying arm or leg

ANIMAL AND EXTREMITY	MAXIMAL PARALYSIS	TOTAL CELLS COUNTED	EXPECTED NO IN SAMPLE	ESTIMATED DESTROYED PLUS ABNORMAL	% OF EX- PECTED NO			% OF REMAINING CELLS						
					Normal	Abnormal	Estimated Destroyed	Diffuse Chromatolysis (Regressive)		Central Chroma- tolysis (Recov- ery)		Necrotic	Normal	
								Mild	Severe	Mild	Severe			
959														
R Arm	5	422†	442	0	100	0	0	0	0	0	0	0	0	100
L Arm	5	451†	451	216	52	48	0	28	0	15	8 (4 9)*	0	0	4 2 52 0
B474														
R Arm	5	423	502	188	62	22	16	9	7	12	3 (5 0)	0	0	3 8 74 2
L Arm	5	453	502	102	80	10	10	7	5	2	2 (1 1)	0	0	2 0 88 3
B8														
R Arm	5	454	537	165	69	15	16	7	0	6	6 (1 5)	0	0	4 4 82 0
L Arm	5	423	537	187	65	14	21	6	4	5	4 (1 4)	0	0	5 4 82 8
R Leg	5	483	526	107	80	12	8	7	3	5	0 (2 3)	0	0	1 0 86 7
L Leg	5	482	526	79	85	7	8	5	6	0	6 (0)	0	0	1 0 92 8
B12														
R Arm	5	449	591	338	43	33	24	8	2	15	8 (8 9)	0	0	19 6 56 4
L Arm	5	437	591	337	43	31	26	5	7	13	5 (8 9)	0	0	22 7 58 1
R Leg	5	551	627	250	60	28	12	10	9	8	8 (5 4)	0	0	11 8 68 5
L Leg	5	556	627	148	77	12	11	7	1	4	5 (2 7)	0	0	2 3 86 1
B22														
R Arm	5	464	552	325	41	43	16	26	7	17	9 (6 5)	0	0	6 5 48 9
L Arm	5	324	552	519	6	53	41	35	2	37	3 (10 7)	0	0	17 3 10 2
R Leg	5	423	475	353	26	63	11	36	4	22	7 (9 5)	0	0	12 1 28 8
L Leg	5	448	475	303	36	58	6	34	8	18	1 (5 4)	0	0	8 7 38 4
Mean		455	538	241	55	30	15	15	8	12	4 (4 9)	0	0	8 2 63 6

† This side not included in calculations because entirely normal

‡ Complete count of single focus in 7th cervical segment

* Figures in parentheses in this and subsequent tables represent percentages of severely chromatolytic cells which are completely chromatolysed or almost so

As was previously shown (Bodian and Cumberland, '47), the pathological process very commonly has its onset in rhesus monkeys on the

TABLE 4
Early Acute Period (1st day)

Analysis of types of neuron damage in samples of motoneuron population supplying arm or leg

ANIMAL AND EXTREMITY	MAXIMAL PARALYSIS	TOTAL CELLS COUNTED	EXPECTED NO IN SAMPLE	ESTIMATED DESTROYED PLUS ABNORMAL	% OF EXPECTED NO			% OF REMAINING CELLS					
					Normal	Abnormal	Estimated Destroyed	Diffuse Chromatolysis (Regressive)		Central Chromatolysis (Recovery)		Necrotic	Normal
								Mild	Severe	Mild	Severe		
A696													
R Arm	5	364	444	153	66	16	18	9	1	7	1 (1 6)	0	80
L Arm	5	436	444	85	81	17	2	12	1	3	2 (0 5)	0	82
R Leg	5	377	483	412	15	63	22	39	6	32	8 (9 0)	0	18
L Leg	4	313	483	416	14	51	35	32	9	33	6 (12 5)	0	21
C725													
R Arm	2	288	484	478	1	58	41	9	7	42	3 (38 2)	0	2
L Arm	2	408	484	446	8	76	16	13	0	63	8 (54 7)	0	9
B26													
R Arm	4	350	514	423	18	50	32	26	6	30	3 (12 9)	0	26
L Arm	5	413	514	435	15	65	20	28	4	31	9 (12 3)	0	19
R Leg	5	425	499	309	38	47	15	41	0	10	1 (5 2)	0	44
L Leg	5	418	499	323	35	49	16	36	4	12	4 (4 3)	0	42
B11													
R Arm	0	47	512	512	0	9	91	4	3	76	6 (53 2)	0	0
L Arm	3	218	512	508	1	42	57	23	4	47	2 (20 6)	0	1
R Leg	5	425	563	370	34	41	25	40	4	8	7 (3 1)	4	45
L Leg	5	423	563	512	9	66	25	19	2	32	9 (21 5)	0	12
B29													
R Arm	3	362	543	506	7	60	33	30	1	26	5 (14 4)	0	10
L Arm	5	416	543	364	33	44	23	38	7	13	0 (4 6)	0	43
R Leg	5	385	515	283	45	30	25	27	0	6	5 (3 1)	0	60
L Leg	5	462	515	182	65	25	10	27	5	0	4 (0)	0	72
Mean		363	506	373	27	45	28	25	5	26	6 (15 1)	0	32

day preceding onset of paralysis At this time in the preparalytic period there is already apparent a sharp decline in the proportion of normal cells This decline continues at about the same rate until the

TABLE 5

Acute Period (2-3 days)

Analysis of types of neuron damage in samples of motoneuron population supplying arm or leg

ANIMAL AND EXTREMITY	MAXIMAL PARALYSIS	TOTAL CELLS COUNTED	EXPECTED NO IN SAMPLE	ESTIMATED DESTROYED PLUS ABNORMAL	% OF EXPECTED NO			% OF REMAINING CELLS						
					Normal	Abnormal	Estimated Destroyed	Diffuse Chromatolysis (Regressive)			Central Chromatolysis (Recovery)		Necrotic	Normal
								Mild	Severe		Mild	Severe		
A816 (2 d)														
R Arm	1	44	402	402	0	11	89	9 1	77 2 (38 6)	0	0	13 7	0	
L Arm	1	127	402	401	0	31	69	14 2	72 4 (40 9)	0	0	12 6	0 8	
C924 (2 d)														
R Arm	3	462	558	483	13	70	17	25 8	57 6 (24 0)	0	0	0 4	16 2	
L Arm	3	474	558	411	26	59	15	23 6	45 3 (13 9)	0	0	0	31 1	
R Leg	1	190	658	658	0	29	71	2 1	83 7 (25 3)	0	0	14 2	0	
L Leg	1	139	658	658	0	21	79	3 6	77 7 (20 1)	0	0	18 7	0	
C817 (2 d)														
R Arm	0	50	532	532	0	9	91	12 0	78 0 (32 0)	0	0	10 0	0	
L Arm	2	278	532	526	1	52	48	29 8	62 2 (12 2)	0	0	5 8	2 2	
R Leg	2	226	568	568	0	40	60	20 8	68 6 (34 5)	0	1 3	9 3	0	
L Leg	3	258	568	551	3	42	55	26 4	54 6 (23 2)	0	0 4	12 0	6 6	
A794 (3 d)														
R Arm	3	416	600	563	6	63	31	14 6	43 3 (35 6)	9 4	23 8	0	8 9	
L Arm	3	534	600	561	7	82	11	23 4	41 2 (18 7)	7 3	20 6	0 2	7 3	
C927 (3 d)														
R Arm	2	358	532	529	0	67	33	6 7	60 3 (30 7)	4 2	27 7	0 3	0 8	
L Arm	2	305	532	532	0	57	43	1 3	66 3 (37 7)	1 3	30 1	1 0	0	
R Leg	1	228	559	559	0	41	59	0	84 2 (64 0)	0	15 4	0 4	0	
L Leg	1	237	559	559	0	42	58	0	90 7 (64 1)	0	9 3	0	0	
C947 (3 d)														
R Arm	3	455	503	479	5	86	9	34 1	56 5 (24 2)	2 2	1 5	0 4	5 3	
L Arm	3	364	503	433	14	58	28	43 2	31 6 (16 2)	2 7	1 6	1 6	19 3	
R Leg	1	250	521	521	0	48	52	2 4	87 8 (62 4)	0	0 8	8 0	0	
L Leg	1	306	521	521	0	59	41	8 5	79 8 (48 1)	1 9	1 3	8 5	0	
Mean		285	543	522	4	48	48	15 1	65 9 (33 3)	1 5	6 7	5 9	4 9	

TABLE 6

Late Acute Period (4-6 days)

Analysis of types of neuron damage in samples of motoneuron population supplying arm or leg

ANIMAL AND EXTREMITY	MAXIMAL PARALYSIS	TOTAL CELLS COUNTED	EXPECTED NO IN SAMPLE	ESTIMATED DESTROYED PLUS ABNORMAL	% OF EX- PECTED NO			% OF REMAINING CELLS						
					Normal	Abnormal	Estimated Destroyed	Diffuse Chromatolysis (Regressive)		Central Chroma- tolysis (Recov- ery)		Necrotic	Normal	
								Mild	Severe	Mild	Severe			
C944 (4 d)														
R Arm	3	440	526	517	2	82	16	14 8	30 0 (9 1)	14 5	38 20	5	2 0	
L Arm	2	282	526	525	0	53	47	6 4	25 7 (24 5)	9 2	37 90	4	0 4	
R Leg	1	313	565	565	0	55	46	11 2	83 4 (36 1)	0 6	3 81	0	0	
L Leg	1	228	565	565	0	40	60	3 9	93 5 (43 9)	0	2 60	0	0	
D81 (4 d)														
R Arm	2	476	583	578	1	81	18	14 5	72 9 (29 0)	4 2	6 70	6	1 1	
L Arm	1	250	583	583	0	43	57	4 8	86 4 (56 0)	0 4	7 60	8	0	
R Leg	1	135	511	511	0	26	74	2 2	84 4 (71 8)	0	12 60	8	0	
L Leg	0	94	511	511	0	18	82	0	79 8 (70 3)	0	20 20	0	0	
C943 (5 d)														
R Arm	3	431	522	509	3	80	17	5 1	35 7 (28 8)	13 0	42 70	5	3 0	
L Arm	2	357	522	505	3	65	32	6 2	32 1 (23 2)	19 3	37 00	6	4 8	
R Leg	1	242	520	520	0	47	53	1 7	60 8 (45 5)	3 3	34 20	0	0	
L Leg	0	210	520	520	0	40	60	0	91 0 (81 0)	0 5	6 61	9	0	
B332 (6 d)														
R Arm	3	318	448	444	1	70	29	17 3	70 1 (23 2)	3 1	7 90	3	1 3	
L Arm	2	277	448	448	0	62	38	1 1	80 2 (53 9)	0 1	15 13	6	0	
R Leg	4	414	469	437	7	81	12	27 0	41 8 (7 5)	6 8	16 40	2	7 8	
L Leg	3	363	469	463	1	76	23	12 9	67 3 (15 7)	4 4	12 90	8	1 7	
B338 (6 d)														
R Arm	3	213	520	503	3	38	59	1 4	17 3 (11 7)	26 8	46 00	5	8 0	
L Arm	5	360	520	448	14	55	31	2 8	6 9 (4 4)	35 0	35 00	3	20 0	
R Leg	4	316	477	440	8	58	34	5 1	50 1 (39 6)	16 4	16 10	6	11 7	
L Leg	5	406	477	365	23	62	15	1 7	25 9 (21 7)	31 3	13 50	0	27 6	
Mean		306	514	498	3	57	40	7 0	57 7 (34 8)	9 4	20 70	7	4 5	

TABLE 7

Subacute Period (7-12 days)

Analysis of types of neuron damage in samples of motoneuron population supplying arm or leg

ANIMAL AND EXTREMITY	MAXIMAL PARALYSIS	TOTAL CELLS COUNTED	EXPECTED NO IN SAMPLE	ESTIMATED DESTROYED PLUS ABNORMAL	% OF EXPECTED NO			% OF REMAINING CELLS					
					Normal	Abnormal	Estimated Destroyed	Diffuse Chromatolysis (Regressive)		Central Chromatolysis (Recovery)		Necrotic	Normal
								Mild	Severe	Mild	Severe		
B339 (7 d)													
R Arm	2	375	568	464	18	48	34	0	3 2 (3 2)	41	128	0	27 7
L Arm	1	225	568	519	9	31	60	0	7 6 (7 6)	37	332	4	21 8
R Leg	1	322	582	575	1	54	45	0	32 3 (32 3)	16	448	8	2 2
L Leg	2	602	582	535	11	89	0	0	6 2 (6 2)	28	254	5	11 1
B935 (8 d)													
R Arm	3	560	855	684	20	45	35	0	8 0 (8 0)	28	830	4	30 5
L Arm	2	488	855	671	21	35	43	0	8 4 (8 4)	23	622	3	37 7
R Leg	0	111	868	842	3	10	87	0	12 6 (12 6)	43	211	7	123 4
L Leg	1	299	868	800	8	27	65	0	10 0 (10 0)	32	832	1	22 8
B32 (10 d)													
R Arm	3	546	775	362	53	17	30	0	1 5 (1 5)	13	4 9	2	75 5
L Arm	3	595	775	395	49	28	23	0	0 5 (0 5)	22	213	4	63 9
R Leg	2	466	781	471	40	20	40	0	0 9 (0 9)	19	712	2	66 6
L Leg	2	300	781	644	17	21	62	0	6 7 (6 7)	23	322	6	45 7
B33 (11 d)													
R Arm	3	249	589	405	31	11	58	1 6	0 8 (0 8)	12	910	9	73 8
L Arm	4	381	589	244	59	6	35	1 1	0 0	6	0 2	3	90 6
R Leg	4	207	408	276	33	18	49	0	0 0	20	415	9	63 7
L Leg	5	235	408	258	37	21	42	0	2 1 (2 1)	20	812	8	63 9
B23 (12 d)													
R Arm	4	343	419	115	73	9	18	2 0	0 3 (0 3)	7	0 1	8	88 6
L Arm	4	295	419	327	22	48	30	12 9	48 9 (17 3)	2	7 4	4	31 1
R Leg	2	181	380	313	18	30	52	11 0	5 6 (3 9)	15	530	9	37 0
L Leg	4	290	380	277	43	33	24	10 3	6 9 (1 0)	16	210	3	56 3
Mean		354	623	459	28	30	42	1 9	8 2 (6 2)	21	620	3	46 7

TABLE 8

Subacute Period (14-22 days)

Analysis of types of neuron damage in samples of motoneuron population supplying arm or leg

ANIMAL AND EXTREMITY	MAXIMAL PARALYSIS	PARALYSIS TERMINALLY	TOTAL CELLS COUNTED	EXPECTED NO IN SAMPLE	ESTIMATED DESTROYED PLUS ABNORMAL	% OF EX- PECTED NO			% OF REMAINING CELLS											
						Normal	Abnormal	Estimated Destroyed	Diffuse Chroma- tolysis (Regres- sive)		Central Chroma- tolysis (Recovery)		Necrotic	Normal						
									Mild	Severe	Mild	Severe								
A761 (14 d)																				
R Arm	3	3	293	478	226	53	8	39	0	2	7	9	2	0	0		86	1		
L Arm	4	4	360	478	156	67	8	25	1	6	0	6	7	2	1	1	0	89	5	
R Leg	4	4	334	452	145	68	6	26	0	0	7	5	0	6	0		91	9		
L Leg	4	4	269	452	210	54	6	40	0	0	4	6	3	3	3	0		90	0	
B167 (15 d)																				
R Arm	4	4	367	568	259	54	10	36	0	1	4	13	6	0	8	0		84	2	
L Arm	4	5	444	568	197	65	13	22	0	0	2	14	8	1	4	0		83	6	
R Leg	3	3	293	516	360	30	27	43	0	0	7	30	7	15	0	0	3	53	3	
L Leg	3	3	279	516	389	25	29	46	0	2	9	28	6	22	2	0	7	45	6	
B97 (16 d)																				
R Arm	1		87	467	385	18	1	81	0	1	2	0	2	3	2	3		94	2	
L Arm	1		125	467	349	25	2	73	0	0	0	1	6	0	4	0		94	4	
R Leg	1		245	331	217	34	40	26	0	21	6	14	3	16	3	1	2	46	6	
L Leg	1		89	331	261	21	6	73	0	1	1	7	9	9	0	3	4	78	6	
A651 (18 d)																				
R Arm	1	3	314	477	167	65	1	34	0	0	3	1	0	0	0			98	7	
L Arm	1	2	165	477	315	34	1	65	0	0	6	0	1	2	0			98	2	
R Leg	1	1	360	523	176	66	3	31	0	0	2	2	1	4	0			96	4	
L Leg	1	1	125	523	318	20	4	76	0	0	13	6	2	4	0			84	0	
B35 (21 d)																				
R Arm	1	1	171	732	731	23	0	77	0	0	0	6	0	0	0			99	4	
L Arm	1	1	140	732	727	18	1	81	0	0	0	3	6	0	0			96	4	
R Leg	0	0	89	826	822	10	1	89	0	0	2	3	2	3	0			95	4	
L Leg	0	0	125	826	816	14	1	85	0	0	2	4	5	6	0			92	0	
C198 (22 d)																				
R Arm	4	5	375	540	187	65	4	31	0	0	5	3	0	5	0			94	2	
L Arm	4	5	356	540	198	63	3	34	0	0	3	9	0	0	0			96	1	
R Leg	4	5	384	566	219	61	7	32	0	0	8	9	0	8	0			90	3	
L Leg	4	5	318	566	265	53	3	44	0	0	5	1	0	3	0			94	6	
Mean			254	540	337	42	8	50	0	1	1	7	7	8	3	8	0	6	86	4

TABLE 9

Early Convalescent Period (35-43 days)

Analysis of types of neuron damage in samples of motoneuron population supplying arm or leg

ANIMAL AND EXTREMITY	MAXIMAL PARALYSIS	PARALYSIS TERMINALLY	TOTAL CELLS COUNTED	EXPECTED NO IN SAMPLE	ESTIMATED DESTROYED PLUS ABNORMAL	% OF EXPECTED NO			% OF REMAINING CELLS					
						Normal	Abnormal	Estimated Destroyed	Diffuse Chromatolysis (Regressive)		Central Chromatolysis (Recovery)		Necrotic	Normal
									Mild	Severe	Mild	Severe		
B190 (35 d)														
R Arm	5	5	667	811	145	82	0	18	0	0	0 2	0	0	99 8
L Arm	5	5	591	811	224	72	1	27	0	0	0 7	0	0	99 3
R Leg	2	5	524	755	243	68	2	30	0	0	2 3	0	0	97 7
L Leg	1	3	306	755	462	39	2	59	0	0	3 9	0 3	0	95 8
A890 (38 d)														
R Arm	2	4	289	514	230	55	1	44	0	0	1 7	0	0	98 3
L Arm	2	4	248	514	267	48	0	52	0	0	0 4	0	0	99 6
R Leg	1	3	195	553	360	35	0	65	0	0	1 3	0	0	98 7
L Leg	1	3	224	553	334	40	1	59	0	0	2 2	0	0	97 8
A690 (38 d)														
R Arm	1	2	165	486	325	33	1	66	0	0	1 8	0 6	0	97 6
L Arm	2	4	228	486	261	46	1	53	0	0	1 3	0	0	98 7
R Leg	1	3	274	474	211	56	2	42	0	0	2 9	1 1	0	96 0
L Leg	1	2	82	474	394	17	0	83	0	0	1 2	1 2	0	97 6
A876 (41 d)														
R Arm	3	4	331	516	189	63	1	36	0	0	1 2	0	0	98 8
L Arm	3	4	353	516	165	68	0	32	0	0	0 3	0 3	0	99 6
R Leg	1	2	186	534	356	33	2	65	0	0	4 3	0	0	95 7
L Leg	1	2	259	534	283	47	2	51	0	0	2 7	0 4	0	96 9
B188 (43 d)														
R Arm	2	4	341	522	192	63	2	35	0	0	2 9	0 3	0	96 8
L Arm	1	4	375	522	154	71	1	28	0	0	1 6	0 3	0	98 1
R Leg	2	4	374	529	173	67	4	29	0	0	4 5	0 3	0	95 2
L Leg	1	2	146	529	396	25	3	72	0	0	6 8	2 1	0	91 1
Mean			308	569	268	52	1	47	0	0	2 2	0 3	0	97 5

TABLE 10

Early Convalescent Period (45-49 days)

Analysis of types of neuron damage in samples of motoneuron population supplying arm or leg

ANIMAL AND EXTREMITY	MAXIMAL PARALYSIS	PARALYSIS TERMINALLY	TOTAL CELLS COUNTED	EXPECTED NO IN SAMPLE	ESTIMATED DESTROYED PLUS ABNORMAL	% OF EX- PECTED NO			% OF REMAINING CELLS						
						Normal	Abnormal	Estimated Destroyed	Diffuse Chroma- tolysis (Regres- sive)		Central Chroma- tolysis (Recovery)		Necrotic	Normal	
									Mild	Severe	Mild	Severe			
B186 (45 d)															
R Arm	4	4	269	526	274	50	1	49	0	0	1 5	0 4	0	98 1	
L Arm	4	5	334	526	204	62	1	37	0	0	1 5	0 3	0	98 2	
R Leg	1	3	104	520	424	18	2	80	0	0	4 8	2 9	0	92 3	
L Leg	1	3	181	520	340	35	0	65	0	0	0 6	0	0	99 4	
B187 (45 d)															
R Arm	1	4	318	572	259	55	1	44	0	0	1 6	0	0	98 4	
L Arm	1	1	126	572	450	21	1	78	0	0	2 4	0 8	0	96 8	
R Leg	1	3	207	517	324	37	3	60	0	0	5 8	1 0	0	93 2	
L Leg	0	0	61	517	462	11	1	88	0	0	8 2	1 6	0	90 2	
B189 (45 d)															
R Arm	2	5	335	493	162	67	1	32	0	0	1 2	0	0	98 8	
L Arm	0	0	92	493	405	18	1	81	0	0	3 3	1 1	0	95 6	
R Leg	1	3	231	530	307	42	2	56	0	0	3 5	0	0	96 5	
L Leg	1	4	286	530	265	50	4	46	0	0	6 3	1 1	0	92 6	
B21 (49 d)															
R Arm	3	5	294	520	228	56	1	43	0	0	0 7	0	0	99 3	
L Arm	4	5	347	520	177	66	1	33	0	0	1 2	0	0	98 8	
R Leg	2	3	135	529	407	23	3	74	0	0	7 4	2 2	0	90 4	
L Leg	3	5	242	529	290	45	1	54	0	0	1 2	0	0	98 8	
B14 (49 d)															
R Arm	3	4	250	495	249	50	1	49	0	0	1 2	0 4	0	98 4	
L Arm	4	5	315	495	185	63	1	36	0	0	1 3	0 3	0	98 4	
R Leg	4	5	250	420	176	58	1	41	0	0	2 0	0 4	0	97 6	
L Leg	4	5	326	420	103	76	2	22	0	0	2 5	0 3	0	97 2	
Mean			235	512	285	45	1	54	0	0	2 9	0 6	0	96 5	

second and third days after onset of paralysis, when only about 4% of the total numbers remain normal in appearance, on the average. Similarly low values are maintained through the fifth day, following which a gradual increase in the proportion of normal cells occurs, reaching a plateau in the period between 3 and 7 weeks after onset.

It is interesting that the peak in the proportion of abnormal cells occurs at the time of the lowest values for normal cells, and that a

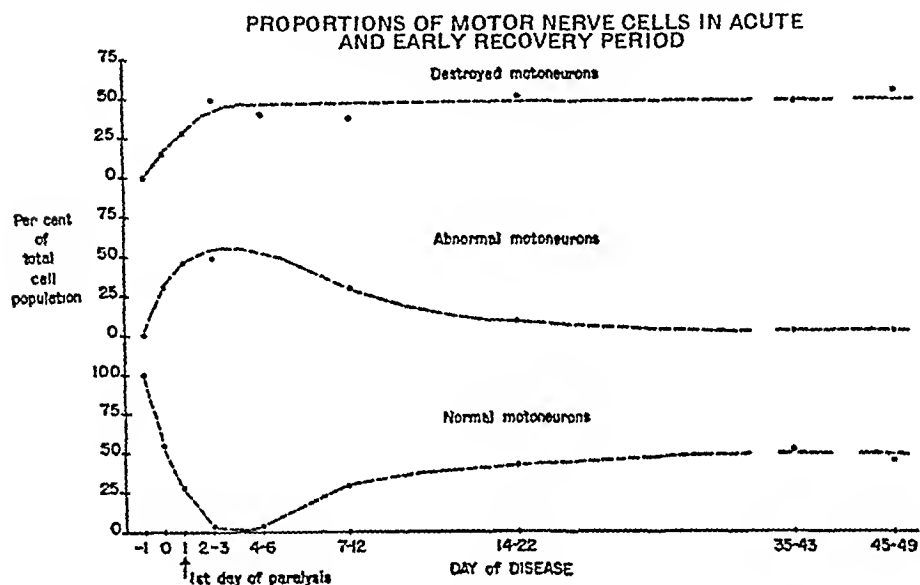


CHART 2 THE CHANGING CHARACTER OF THE MOTONEURON POPULATION AS INDICATED BY THE MEAN CELL COUNTS AT DIFFERENT STAGES

Each point represents the mean of the motoneuron populations supplying 20 limbs, with an average severity of paralysis of grade 2 (moderate to severe paralysis). Data from Tables 3 to 10. Values for destroyed motoneurons obtained by subtraction of normal and abnormal motoneurons from the total expected number.

steady decline follows which leads to negligible values when the normal plateau occurs. It is important to note that the plateau for destroyed cells is reached rather abruptly, and at a time coincident with peak values for abnormal cells and lowest values for normal cells. This leads to the inescapable conclusion that no appreciable increment of destroyed cells occurs after this time. Moreover, the increase in normal cells after the second to fifth days must occur by the recovery of abnormal cells.

Changing proportions of normal cells, and abnormal stages with respect to the total number of remaining cells In order to obtain more detailed information about the stages of regression and of recovery in infected cells, the changing proportion of normal cells and abnormal stages with respect to the total number of remaining, undestroyed cells was analysed. It was intended that such an analysis should show the progression of cytopathological changes by revealing the time when each stage of cell change reaches its peak in terms of proportion of the remaining cell population. Destroyed neurons were not included, since this portion of the population is essentially negligible after the third day.

In order to classify the pathological stages to be included in the statistical study, a preliminary survey was carried out, based on the following assumptions:

- 1 The regressive and recovery changes produced by virus action in nerve cells are progressive and follow essentially the same sequence in all cells, although the rates of change may be presumed to be variable.

- 2 Pathological stages present in large numbers in the acute period of the disease and rare or absent in later periods, represent injury stages.

- 3 Pathological stages present in large numbers in the subacute and convalescent periods, and rare or absent in the acute stage, represent recovery stages.

The morphological intracellular changes in motoneurons fortunately were sufficiently distinctive in character, as shown in the first section, so that they could be classified in a relatively simple manner. The adequacy of the classification in terms of the above assumptions and of the dynamics of the disease itself appeared to be satisfactorily confirmed by the results to be described. It was found that the predominant cytopathological change in remaining neurons in the earliest pre-paralytic cases was a diffuse cytoplasmic chromatolysis, largely mild in degree (Table 3, Chart 3, Plate 13, fig. 1). In the early acute paralytic period diffuse chromatolysis of a severe degree increased in prominence, and reached peak proportions between 2 and 5 days. (Severe diffuse chromatolysis, Chart 3, Tables 5 and 6, Plate 13, fig. 2).

Neurons with diffuse cytoplasmic chromatolysis short of complete absence of Nissl bodies were rare after the acute stage, so that progressive diffuse thinning of Nissl bodies may be assumed to identify the regressive

stages due to virus action After the first 3 days of paralysis, during which diffusely chromatolytic and "necrotic" cells dominate the cytopathological picture, a new morphological appearance of neurons is seen in considerable numbers of cells for the first time This is the appearance of "central chromatolysis", characterized by an increased density of Nissl bodies in the cell periphery and near the nuclear membrane, but

PROPORTIONS OF NORMAL AND ABNORMAL MOTONEURONS IN ACUTE AND EARLY RECOVERY PERIOD

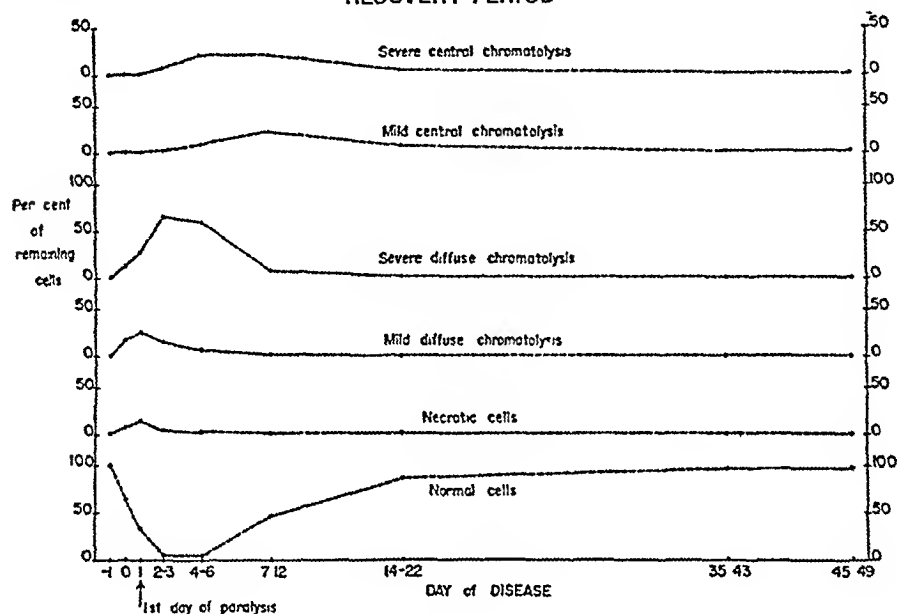


CHART 3

The changing character of the motoneuron population, expressed in proportion of cells in each pathological stage to the total number of remaining cells at each period of the disease Data in Tables 3 to 10

with rarefied or absent Nissl substance in the intervening cytoplasm (Plate 13, fig 3) Whereas the nucleus is generally centrally located in the cell body in cells showing diffuse chromatolysis, it is more apt to be eccentrically displaced in cells showing "central" chromatolysis The cells showing central chromatolysis are at first relatively few in number and mixed with cells showing diffuse chromatolysis (Tables 5 and 6) By the 7th day after onset of paralysis, however, the latter are rather rare, whereas the former represent the principal pathological cell type except for a few necrotic forms (Chart 3, Plate 13, fig 4) In

progressively later stages, the Nissl substance is seen in greater amounts in such cells. Thus, the appearance of central chromatolysis with progressively increasing Nissl substance may be assumed to identify the recovery changes in nerve cells injured by virus action. It is interesting that central chromatolysis is first seen when paralysis has ceased to progress and persists in diminishing number of cells, becoming negligible in number after the 3rd or 4th weeks.

Cells which are severely chromatolysed in the acute stage, probably pass through the stage of severe central chromatolysis first, and later that of mild central chromatolysis, before complete recovery of chromophil substance. On the other hand, it is likely that many cells are only lightly chromatolysed at first, and only pass through the stage of mild central chromatolysis before complete recovery. This likelihood seems to be suggested also by the quantitative data showing that the peak proportions of cells showing mild and severe central chromatolysis, respectively, are not sequential but concurrent in time, with the mild stages persisting over a longer period (Chart 3).

III Damage and Recovery of the Nerve Cell Population as Related to Motor Paralysis and Recovery

The previous sections have illustrated the possibility of studying the injury and recovery changes in the motor nerve cell population in sufficient quantitative detail to make feasible a formulation of several principles which describe rather complex pathological phenomena in a relatively simple manner. The relating of these cellular phenomena to the clinical concepts of paralysis and recovery from paralysis is a matter of much greater difficulty. First of all, the clinical symptom "paralysis" is not easily defined in measurable terms. The normal function of a limb is an exceedingly complex process, in which many parts of the nervous system take part in determining the movement of muscle and bone. The precise timing of a complex sequence of movements and the coordination of action of antagonistic and synergistic muscles are effected by the function of centers in the cerebral cortex, basal ganglia, brainstem, cerebellum, and spinal cord, in response to "voluntary" initiation, and to sensory reflex discharges frequently emanating from the muscles themselves. The central nervous mechanism, however, must exert any influence upon the muscles by acting

through the motoneurons, which constitute the "final common pathway" of CNS motor discharges. Thus, although destruction or damage of motoneurons may be directly productive of loss of muscle function, injury to higher motor centers, which have been shown to be affected in variable degree in poliomyelitis (Bodian, '47), can only be apparent clinically if the "lower" motoneurons are functioning normally. As it happens, the predominant injury in poliomyelitis occurs in the motoneuron population of the spinal cord, so that damage to higher centers either is masked as a rule, or is often too minor in degree to be manifest clinically. Nevertheless, the fact that impaired motor function may result from injury to centers other than lower motor neuron pools makes it difficult at times to assess accurately the degree of weakness or "paralysis" in poliomyelitis. For example, the spasticity of early poliomyelitis in monkeys may be so extreme as to make it difficult for animals to climb, although motoneurons are quite intact (Bodian, '47). Manual examination of extremities in more detail, however, reveals no lack of power, but only muscular rigidity as the limiting factor in movement. The rigidity, of course, disappears in those muscles where paralysis due to lower motor neuron damage becomes severe. It is thus evident that loss of muscular power rather than inadequacy of motor performance is the best index of motoneuron damage in the acute stage, and in later stages atrophy of muscles can be used as a confirmatory sign of motoneuron destruction.

The total potential power of contraction is normally rarely called into play, and in fact it is obvious that only a small fraction of the potential muscle power is necessary for ordinary motor activities. When a measure of motor function is desired, this is usually obtained by assessing the potential power of muscles or muscle groups by means of ergographs or by means of less accurate arbitrary standards which involve the degree of performance against the resistance of gravity. Since the available power of normal muscles is determined by the general physical condition of the individual, and varies with fatigue, exercise, nutritional state, etc., it is obvious that the motor function of the CNS, or even of the muscle itself cannot be accurately quantitated over a period of time without control of these factors. When all these factors are considered, the potential power of some single muscles or of single synergistic muscle groups can be measured with a fair degree of

accuracy Unfortunately, however, the motoneurons supplying individual muscles or small muscle groups cannot be accurately localized in the spinal cord except by experimental means, and their localization in an infected spinal cord is even more difficult As we have earlier indicated, the motoneuron population supplying an entire limb can be more readily identified, and a reasonably accurate sample of the population can be counted and classified into pathological stages We have therefore accepted the difficulty of accurately assessing the motor function of an entire limb and have used a system of grading which is not over-refined but which appears to be adequate for our purposes The method has been described in one of the introductory paragraphs of this report It must be emphasized here, however, that the grades of function are arbitrary and represent quantities of increasing magnitude but of unknown value

The Role of Motoneuron Destruction

In view of the relative crudeness of muscle testing, the first question which must be asked is how closely can motor function be correlated with motoneuron destruction in the early convalescent period At this time, between about one and three months after onset, the simplest possible relation between the size of the remaining motoneuron population and the degree of residual weakness may be obtained, since most of the remaining neurons are normal in appearance, and probably in function Moreover, hypertrophy of muscles, which may reach considerable proportions in partly paralysed limbs after the third month, has not as yet had a chance to affect the relation between residual motoneurons and residual muscle function to too great a degree It is not uncommon, for example, to find an increase in mass of muscle of a partly paralysed limb from about one-half of that of its normal mate to almost normal dimensions between the third and sixth month

Table 12 shows the relation between degree of paralysis and the percentage of surviving motoneurons of 78 paralysed limbs from 20 animals (Tables 8 to 11) killed between three weeks and three months after onset of paralysis (see also Table 15, D, and Chart 4, D) The average grade of severity of paralysis of all limbs was roughly 2 at the height of paralysis, and 3 at the time the animals were killed In all of these animals the surviving motoneurons were all or almost all normal

TABLE 11

Relation of Paralysis to Destroyed Motoneurons in Convalescent Period

ANIMAL AND EXTREMITY	DURATION OF PARALYSIS IN DAYS	MAXIMAL PARALYSIS IN ACUTE STAGE	PARALYSIS TERMINALLY	TOTAL CELLS COUNTED	EXPECTED NO	% ESTIMATED DESTROYED
C903	31					
R Arm		1	2	174	500	65
L Arm		1	2	229	500	54
R Leg		0	0	0	600	100
L Leg		0	0	0	600	100
C437	57					
R Arm		1	3	173	442	61
L Arm		1	2	89	442	80
R Leg		0	0	53	437	88
L Leg		0	0	19	437	96
A505	75					
R Arm		2	4	244	461	47
L Arm		2	4	242	461	48
R Leg		1	1	42	317	87
L Leg		1	1	116	317	63
A506	72					
R Arm		1	2	126	440	71
L Arm		1	2	133	440	70
R Leg		1	1	51	258	80
L Leg		1	2	57	258	78
A713	84					
R Leg		1	2	79	513	85
L Leg		1	5	326	513	37
A714	84					
R Arm		2	5	293	549	47
L Arm		2	5	381	549	31
R Leg		1	5	302	505	40
L Leg		1	5	343	505	32
A956	93					
R Arm		4	4	247	575	57
L Arm		4	5	379	575	34
R Leg		0	1	113	745	85
L Leg		0	1	60	745	92
A394	101					
R Arm		0	0	64	565	89
L Arm		4	4	319	565	44
R Leg		5	5	392	516	24
L Leg		4	4	239	516	54

in appearance The mean and the range of the percentages of surviving cells are shown in the motoneuron population of limbs of specified grade at the time of death Of special interest are the values for

TABLE 12

Relation between Severity of Paralysis and Destroyed Motoneurons in Early Convalescent Period†

CLINICAL GRADE WHEN KILLED‡	PER CENT OF MOTONEURONS DESTROYED		NO LIMBS
	Mean	Range	
5	34	18-54	22
4	44	28-57	17
3	62	42-80	10
2	70	52-85	12
1	80	63-92	8
0	91	81-100	9
Total			78

† Animals killed 3 weeks to 3 months after onset

‡ Average clinical grade at height of paralysis—about 2

Average clinical grade when killed—about 3

TABLE 13

Relation between Severity of Paralysis at its Height and Destroyed Motoneurons†

CLINICAL GRADE AT HEIGHT OF PARALYSIS‡	PER CENT OF MOTONEURONS DESTROYED		NO LIMBS
	Mean	Range	
5	28	18-43	4
4	35	18-57	24
3	41	23-58	12
2	42	0-74	18
1	62	26-87	44
0	90	81-100	12
Total			114

† Animals killed 7 days to 3 months after onset

‡ Average clinical grade at height of paralysis—about 2

limbs graded as normal, and as completely paralysed (5 and 0), since these clinical grades, and especially the latter, are the most accurately determined It will be noted that of 22 limbs graded as "normal" in

function at the time of death, none showed less than 18% destruction of motoneurons, and the average value was 34%. It, therefore, appears that as much as a third of the motoneuron population may be destroyed without detection of weakness of the limb by our method of examination, which includes observation of climbing, running, or jumping, careful manual examination, and inspection for evidence of atrophy (see also Pette, Demme, and Kornyei, '32, page 245). Our earlier observations suggest that this figure for "normal" limbs may be high for many non-paralytic cases, but it is not high for paralytic cases regardless of the "mildness" of the paralysis. Of the 22 limbs graded as "normal" between the first and third months, 3 were never noted to be paralysed, 10 were paralysed to the degree 4 at the height of paralysis, and the remaining were distributed among grades 3, 2 and 1 at the height of paralysis.

At the other extreme it was found that 9 of 11 limbs completely paralysed in the acute stage, or showing only traces of muscular activity, failed to recover any function whatever. This agrees with general experience in human cases, in which it is found that the possibility of any recovery whatever is very low in extremities completely paralysed in the acute stage. In only 2 of our 9 cases of total limb paralysis, however, was the destruction of motoneurons complete in the sections examined. The average loss was 91%, which suggests that a residual population of about 10% of motoneurons is incapable of producing clinically observable movement in some cases. In other cases, graded as 1 when killed, a residual population no larger or very little larger was capable of producing movement because it was concentrated largely in an area supplying a small muscle group, usually the toe or finger flexors, which often was the last group to be involved.

The proportion of destroyed motoneurons in the intervening grades (4 to 1), at the time the animals were killed, shows a good correspondence with the degree of paralysis. Although there is considerable overlap in the ranges of adjacent groups, this is not surprising in view of the difficulties of clinical grading and the role of variation in distribution of destruction, to be discussed. The demonstration of a general proportionality between motoneuron destruction and degree of paralysis in the early convalescent period is, of course, confirmatory of an established fact which can be verified by a much easier survey of spinal cord

sections from selected cases. The larger quantitative experience, however, makes possible more accurate analysis of details with an assurance that cannot be obtained by an unsystematic survey of spinal cord sections.

With a degree of confidence in the validity of the method of relating motor function to the status of the motoneuron population, engendered by the above described results, we can now proceed to examine in the same animals the relationship between motoneuron destruction and clinical grade at the height of paralysis in the acute period. The values given in Table 13 show the percentages of surviving motoneurons in animals killed between 3 weeks and 3 months after onset, as related to limbs of specified clinical grade at the time of the height of paralysis in the acute stage. An additional 36 limbs (Tables 7 and 8) from animals killed after the seventh day are added to those of Table 12. Since it was shown in the previous section that negligible death of motoneurons occurs after the height of paralysis is reached, it may be assumed that the number of motoneurons found destroyed at any time after 7 days is the same as that which would have been observed if the animal had been killed at the height of paralysis. It will be noted at once that in animals with the severest grade of paralysis (0) at the height of paralysis, the proportion of destroyed motoneurons is the same as it is in animals with the same clinical grade when killed in the early convalescent period (Table 12), as is to be expected. The limbs in other grades at the height of paralysis, however, show a consistently lower mean value of destroyed motoneurons than limbs of comparable grade in the early convalescent period. In grades 1, 2 and 3 the difference is about 20% in each. This indicates that a sizable component of the paralysis of the acute period cannot be accounted for by motoneuron destruction. That this component may be very large in some cases is strikingly shown by the lowest values in grades from 1 to 5, all of which fall below 26, whereas the lowest values in Table 12 follow a stepwise order from 18 to 63 per cent, consistent with the mean values.

It is plain, therefore, that most of the remaining weakness observed in the early convalescent period can be accounted for on the basis of motoneuron destruction alone, whereas the paralysis observed in the acute stage is also in part the result of other factors. One of these factors is probably the reversible injury of motor nerve cells, since it

was shown in the preceding section that many motoneurons may be severely injured and yet recover, as far as microscopic structure is concerned, before the early convalescent period

The Role of Reversible Motoneuron Injury

With the knowledge that extensive reversible changes occur in motoneurons in the acute period, we have attempted an analysis of their role in the production of the recoverable component of paralysis

TABLE 14

Relation between Severity of Paralysis and Pathological Status of Motor Nerve Cells in Acute Period (0 to 12 days)

CLINICAL GRADE WHEN KILLED	1		2		3		NO LIMBS
	% of Cells Destroyed		% of Cells Destroyed, Necrotic or Completely Chromatolytic		Column 2 Corrected to Include $\frac{1}{2}$ of Cells Showing Severe Chromatolysis†		
	Mean	Range	Mean	Range	Mean	Range	
5	18	0-43	28	5-68	32	12-72	29
4	30	12-49	39	18-60	46	19-69	9
3	31	10-59	44	23-77	56	28-82	18
2	38	0-60	59	36-90	70	45-91	15
1	60	40-89	79	63-95	87	70-97	16
0	82	60-91	94	90-97	96	91-98	5
Total							92

† This gives severely chromatolytic cells the functional value of half as many normal cells

The motoneuron populations of 92 limbs were studied in animals killed between the preparalytic period and 12 days after onset of paralysis. As can be seen in Table 14, column 1, and in Chart 4, A, the average proportion of destroyed cells alone is much less for each grade of clinical function than is the case in the early convalescent period when the morphological status of the motoneuron population is stabilized (Table 12, Chart 4, D). In Table 14, column 2, the proportion of all cells which were considered as very likely to be non-functional is given for limbs of each clinical grade. These cells include estimated destroyed cells, cells which we have previously defined as "necrotic", but which still have recognizable nuclei, and cells which are completely

chromatolysed, that is, have no remaining Nissl substance in the cytoplasm. These proportions, also shown in Chart 4, B, compare well with those found in the "stable" motoneuron population of the early convalescent period (Table 12, Chart 4, D), with the greatest discrepancy seen in limbs of grades 2 and 3. Since it was thought that some cells graded as "severely chromatolytic" could be functionless

RELATION BETWEEN SEVERITY OF PARALYSIS IN ACUTE STAGE (0 TO 12 DAYS) AND PATHOLOGICAL STATUS OF MOTONEURONS

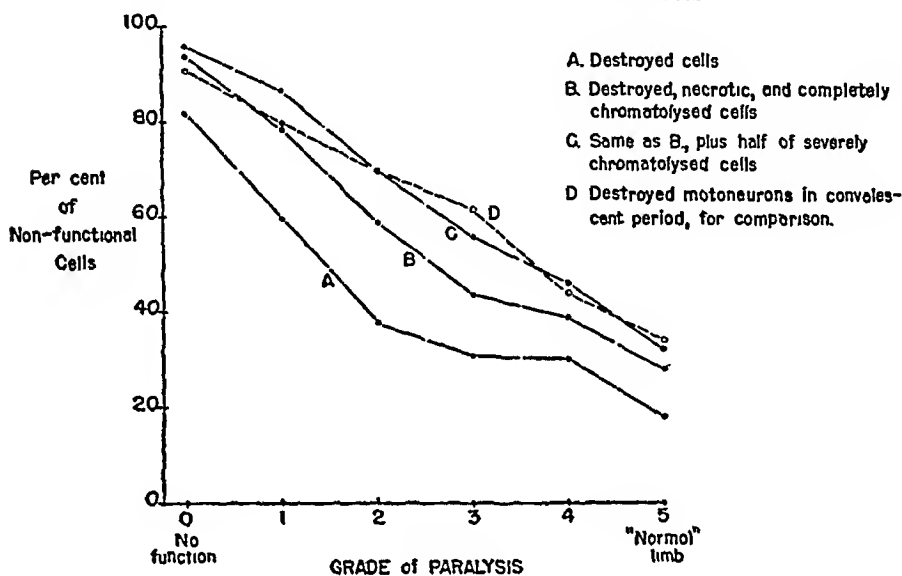


CHART 4

See page 51 for detailed explanation. The lines A, C, and D correspond to the correlation tables in Table 15, and show the trends of mean proportions of "non-functional" motoneurons in relation to functional grades. Since the quantitative value of each clinical grade is unknown, as explained on page 44, the lines are not to be considered as accurate curves, since the slopes are unknown.

The grades of paralysis shown below are explained on page 8. Grade 5 represents normal or almost normal limbs, and grade 0 completely paralysed limbs.

also, this assumption was then tested as well. It was found that when half of such cells were counted as non-functional, and added to those included in Table 14, column 2, a result was obtained which fitted well with the comparison of motoneuron populations and clinical grade of the early convalescent period. (Compare Table 12 and Table 14, column 3, and Chart 4, C and D). This result brought out a fact previously unrecognized by us, namely that severely chromatolysed cells were relatively more numerous in the "middle" grades of paralysis (2 and 3).

The correspondence between lines C and D in Chart 4 is of considerable interest in suggesting that the function of an infected neuron is lost in the stage of injury change which we have described as "severe

TABLE 15

Grade of Limbs in Poliomyelitis Cases in Relation to "Non-Functional" Motoneurons†

GRADE OF LIMBS IN ACUTE STAGE (0-12 D)	0-9	10-19	20-29	30-39	40-49	50-59	60-69	70-79	80-89	90-100	
A % of Cells Destroyed											
5	7	11	8	1	2						r = 0.76
4		2	2	4	1						
3	1	5	5	3		4					
2	1	2		5	5	2	1				
1					3	7	1	3	1		
0						1	1		2	2	
C % of Cells Destroyed, plus Necrotic Cells, Completely Chromatolysed Cells, and $\frac{1}{2}$ of Severely Chromatolytic Cells											
5	3	4	9	6	3	2	1	1			r = 0.82
4			2	3		3	1				
3			1	4	4	3	4	1	1		
2				1	2	3	4	3	2	1	
1							1	5	6	4	
0										5	
D % of Cells Destroyed											
GRADE OF LIMBS IN SUB ACUTE STAGE (21 D-3 MO)											
5		1	4	12	4	1					r = 0.86
4			2	3	8	4					
3					1	4	3	2			
2						2	4	4	2		
1							1	3	3	1	
0									6	3	

† The frequency numerals in the tables refer to the number of limbs in each category. Grade 5 = normal, grade 0 = complete paralysis.

chromatolysis", and that cells in stages of more severe change are functionless. This supposition is in agreement with findings from individual cases described in Section II. Moreover, the great disparity

between values of C and those of A, especially in the "middle" clinical grades, suggests that a large component of the lost function in the acute stage may be due to the reversible injury represented by the stages of severe chromatolysis. Cells in these stages are very numerous between the fifth and twelfth days, when significant numbers of cells are no longer dying off.

Table 15 shows these relationships in the form of a correlation table, with A, C, and D corresponding to the trends in mean values shown in lines A, C, and D of Chart 4. An obviously high degree of correlation between grade of limb function and destroyed motoneurons is shown in the early convalescent period (D), with a correlation coefficient, r , of 0.86². In the acute period the correlation, although high also, is somewhat less (A), with a shift in the direction of less motoneuron destruction in each grade than shown in (D). This means that cell destruction alone cannot completely account for the degree of paralysis observed, although it is an important factor. In (C) the effect of including necrotic cells, completely chromatolysed cells, and half of the severely chromatolysed cells in the category of "non-functional" is shown. The relation between grade of function and proportion of non-functional cells is much closer to that found in the early convalescent period (D). The discrepancy is very likely due in large part to the greater difficulty of accurately assessing grades of function in the acute period. In the latter stages, for example, the presence of asymmetrical atrophy of limbs is a helpful adjunct in the assessing of function and probably prevents certain errors of grading to some extent.

The Role of Distribution of Cell Injury and Destruction

It was expected at the beginning of this study that great difficulty would be encountered in comparing the pathological status of the motoneuron population and the function of the corresponding limb as a whole. Spotty paralysis in a limb is often observed, with obvious paralysis of one muscle group and very little if any, in another group in the same limb. Similarly, one frequently observes severe destruction of motoneurons on one side of a spinal segment, or part of one, and little if any destruction of adjacent segments. Although these occur-

² A correlation coefficient of 1 represents complete association, one of 0 represents no association.

rences are striking, they are by no means the rule, since a relatively homogeneous distribution of pathological effect is also commonly seen. Moreover, in our experience, the spotty distribution of pathological change rarely results in the selection of a single functional muscle group for destruction, but usually has a segmental character, so that alternating regions along the cephalocaudal axis are affected severely or moderately. As is well known, segmental lesions, such as follow spinal root section, produce diffuse and unspectacular weakness because spinal roots of the limb areas supply antagonistic groups, and because no important limb muscle groups are supplied by only one spinal root. Consequently, the function of the limb as a whole has been a surprisingly good average indicator of the state of most of its constituent muscle groups. Moreover, as was shown in Section 2, paralysed animals have such a widespread distribution of motoneuron damage in the entire motoneuron population, that it is hard to believe that any limb muscle group can escape some degree of injury, although some loss of function may be below the clinical threshold.

Nevertheless, of course, single muscles or muscle groups occasionally are hard hit by an unusual localization of neuron damage and destruction, or conversely a muscle or muscle group may be spared to an unusual extent. In the monkey the muscles most commonly spared in otherwise completely paralysed limbs are the flexors of fingers or toes, and a correspondingly intact group of motoneurons can be found in such cases in the posterolateral cell column in the two lowest segments of the spinal cord enlargements. In several convalescent cases we have found such isolated sparing of motoneurons of this group in the brachial region to be associated with severe flexion contracture of the fingers, due to unopposed action of the flexor muscles. Such contractures did not occur in arms corresponding to spinal cord enlargements in which this motor cell group was completely or almost completely destroyed.

It is interesting that Elliott ('45) has confirmed earlier workers in describing a pattern of neuron destruction in convalescent and chronic human cases in which the ventrolateral cell groups of the anterior horn are most commonly spared, and most constant injury occurs in the region of the commissure and in the center of the horn (Blanton, '17). Elliott, however, interprets this as indicating a concentric spread of infection from a dorsomedial point in the cross-section of the spinal

cord From a study of acute cases as early as the preparalytic period one arrives at a rather different interpretation of his findings Although we have observed a tendency for early inflammatory lesions to be located dorsomedially, especially in the vicinity of branches of the anterior spinal vessels, we have seen no suggestion in earliest preparalytic cases that the first neuron lesions are located dorsomedially or spread concentrically As was mentioned in Section 1, the earliest changes in motoneurons, without surrounding inflammatory changes, may be seen at the lateral periphery of the anterior horn or in any other position Spread of virus seems to be so rapid and general that in the early paralytic period most anterior horn cells in the enlargements show evidence of infection Observations made in the chronic stage cannot reveal the generalized character of the infection in the motoneuron population, since remaining neurons are completely recovered as a rule and may seem not to have been involved

In our convalescent and chronic cases the pattern of neuron destruction is quite variable, but there is a tendency for motoneurons in the periphery of the anterior gray columns to have a greater chance of survival Not only the ventrolateral group, emphasized by Elliott as preferentially spared, but also the most medial cells are more often persistent than centrally located cells in the anterior columns of the enlargements This is also Horanyi-Hechst's finding in an extensive study of human cases ('35) Moreover, the neurons bounding the enlargements in the longitudinal plane are also more resistant to destruction than those in the limb areas, as is well known In the monkey, sacral, thoracic and upper cervical motoneurons are in this category (Pette, Demme, and Kornyey, '32) Elliott's concept of concentric spread of lesions from the commissural region must therefore be questioned, since his findings indicate only a tendency of preferential sparing of peripheral neurons rather than the direction of spread of the pathological process

In many cases, not even the above-mentioned pattern of sparing of motoneurons is seen Frequently one observes severe destruction of lateral groups with complete sparing of medial groups, and vice versa Moreover, in some cases foci of extensive cell destruction alternate irregularly with areas of sparing, whereas in others a rather homogeneous destruction of nerve cells is seen with not a single large area of

motoneuron disappearance In such cases, there is no suggestion of a "pattern" of preferential cell destruction

Finally, it is problematical whether the tendency for greater paralysis of some muscle groups and more common sparing of others can be correlated precisely with corresponding tendencies for greater or less "susceptibility" in their motor nerve cell "groups" For example, a given proportion of motoneuron destruction in a functional group with high innervation ratio may produce a quite different result than the same proportion in a group with a low innervation ratio When relatively few motoneurons supply a large muscle, destruction of a given proportion may denervate a relatively large mass of muscle, whereas equivalent destruction in a functional center supplying a small muscle with a high innervation ratio would denervate a relatively small mass Moreover, the greater compactness of a functional motoneuron group may prejudice the chances for survival of a sufficiently large number of cells of a functional pool to produce adequate function Unfortunately our knowledge of nerve-muscle relationships is not sufficient to give all of these factors their proper weight in the determination of the pattern of paralysis

Distribution of Lesions in Non-Paralytic Cases and in Monkeys Infected with "Mild" Strains

In previous reports (Sabin and Ward, '41, Bodian and Howe, '41a) the wide variation in the extent of lesion formation in non-paralytic attacks of poliomyelitis has been described In the mildest of such cases in the rhesus monkey not only may the cerebral lesions be more limited than they are in paralytic cases but also lesions may be found in only a few segments of the spinal cord or may be quite absent in the spinal cord enlargements although present in the hindbrain (Bodian and Howe, '41a, case A922, no lesions in cord segments C5 to T1, and L4 to S1) This is in striking contrast with the paralytic cases described in this report, in which hardly a section in the cord enlargements was free of lesions As a matter of fact, most non-paralytic cases show as extensive distribution of lesions in the spinal cord as do paralytic cases, and differ from the latter not in the number of motor nerve cells invaded but rather in the severity of the injury to invaded nerve cells and in the number destroyed Most non-paralytic cases and cases

with very slight weakness are indistinguishable, as far as cord pathology is concerned, from the part of the cord of "severe" cases which supplies a spared extremity

It is interesting, moreover, that animals infected with "mild" poliomyelitis strains show a similar extensive distribution of lesions and a high proportion of invaded motoneurons. For example, the Frederick strain, isolated from a human throat swab in 1944 in Baltimore (Howe and Bodian, '47), exhibited the mildest paralytic cases in our experience in animals inoculated with virus from the first monkey passage. These animals nevertheless showed lesions differing in no way from those of "severe" strains in extent of distribution or in character, except that invaded cells usually showed less severe cytopathological changes and fewer were destroyed. Inflammatory changes were also much less severe as a rule, as might be expected with less cell destruction. Yet in some sections all motoneurons on one side were destroyed, so that the local picture resembled that seen in animals with prostrating paralysis. In one case (C59) killed 4 days after slight paralysis was suspected in one arm and one leg, and showing no progression of paralysis, only 4% of the expected number of normal cells was found in a sample of 79 sections through all segments of brachial and lumbar cord enlargements. Most of the motoneurons were in the stage of mild chromatolysis, (59% of remaining cells), and 21% of the expected number were destroyed (Plate 14).

The extensive motoneuron invasion with "mild" strains like the Frederick agrees with the finding that a pool of spinal cords from animals inoculated with virus from the first monkey passage gave a titer of about 1×10^{-5} in monkeys inoculated intracerebrally. This compares well with the titers obtained with pools of "severe" strains under the same conditions, and suggests that optimum virus titers, as well perhaps as virus dissemination, in the nervous system is not dependent on extensive destruction of host cells.

DISCUSSION

Cytopathological Changes in Motoneurons The finding of progressive chromatolytic changes in motoneurons in the acute stage agrees with earlier observations made by Hurst ('29) and others, in both monkeys and man. Sabin and Ward ('41) have illustrated the probable se-

quence of changes in a manner similar to that shown in Plate 1 of this report, and from examination of non-paralytic cases have suggested "that a cell attacked by poliomyelitis virus perhaps need not invariably be irreversibly damaged" The quantitative survey of accurately timed stages in the present study leaves no doubt that this occurs, and shows in fact that in some cases a high proportion of cells are chromatolysed and subsequently recover Of special interest is the fact that cells which are chromatolysed by virus activity are either quickly destroyed during the first few days of the disease or undergo slower recuperative changes leading to complete morphological recovery in a period of about 1 month Both Hurst ('29) and Sabin and Ward ('41) have commented upon the normal appearance of most nerve cells after this time, but our larger series of cases shows that this essentially complete recovery of motoneurons is the rule Moreover, the stages of morphological recovery can be identified, using the Nissl substance as an indicator, and studying cell populations quantitatively in a sequence of clinical stages

Comparison of Humans and Chimpanzees with Rhesus Monkeys It is evident that numerous difficulties prevent an analysis of human material in as adequate detail as is permitted in experimental animals Among these, first of all, is the problem of determining the time of onset of pathological changes In monkeys the use of "severe" passage strains produces a fairly abrupt onset, so that it is possible to time the early stages of the disease accurately (Bodian and Cumberland, '47) The onset of the disease in humans is more often insidious, or of the "dromedary" type, and at times no clinical symptom clearly marks a definite stage of the histopathological process Moreover, the important preparalytic stage, and the earliest paralytic stage, is not available in autopsy material Finally, autopsy material is often poorly preserved, so that postmortem changes mask important cytopathological changes

In spite of the afore-mentioned difficulties, a study of the changes in human spinal cords as compared with those worked out in detail in the monkey has proved to be interesting and instructive Our own available material includes the spinal cords of 16 fatal human cases, prepared as much like our experimental material as possible Six of these cases showed such overwhelming destruction of anterior horn cells at

all levels that they were of no value in studying sequential changes in these cells. In the remaining ten cases the disease had had a duration of from 1 to 2 days in the earliest to about 8 to 12 days in the latest, and of these cases only about half showed relatively good postmortem tissue preservation.

Only outstanding features of the histopathological process could be used to compare the disease with that in the monkey. In the earliest human case available (H35) the disease had had a duration of only 1 to 2 days before death ensued. This case was indistinguishable in essential particulars from monkey cases of similar duration. The predominant pathological stage in motoneurons consisted of diffuse chromatolysis, with many cells completely chromatolysed, necrotic, or undergoing early neuronophagia. A similar sequence of changes in motoneurons in a child of 4 years who died 30 hours after onset of the disease was described and well illustrated by Crow ('31). Three cases were available with a disease duration of about three days (H33, H36, H37). In all three cases there was definite recession of the destructive process, as far as the spinal motoneuron population was concerned. Active neuronophagia was less conspicuous, and chromatolytic cells less numerous than in the earlier case. In one of these cases, central chromatolysis, as well as diffuse chromatolysis, was seen in some of the lumbar segments.

It is interesting that active neuronophagia was uncommon in all five of the cases with a duration of disease longer than 3 days. The predominant pathological form seen among motoneurons in these cases was that of central chromatolysis, although numerous necrotic cells were also in evidence. In the case with longest duration (H34), with disease onset about 8 to 12 days before death, the first paralysis was noted 5 days before death. In this case, cells with typical central chromatolysis were abundantly seen, greatly exceeding in number the pathological stage with next greatest frequency of occurrence, namely persistent necrotic forms. Diffusely chromatolytic cells were infrequent in occurrence, and not only neuronophagia but also the cell clusters which persist for a period after neuronophagocytosis has been completed were conspicuously absent. The motoneuron illustrated by Goodpasture ('41) well illustrates this stage of the disease (central chromatolysis—fig 7, page 121).

Although the pathological process in man, including the time course, is remarkably similar to that in the monkey, certain important differences were observed. Except for the fulminating case (H35), the other human cases appeared to be older by one or several days than suggested by the time of recorded onset of paralysis. This suggests that the pathological process in man as a rule does not start as abruptly as in severe monkey cases inoculated with large doses of virus, but perhaps starts earlier than indicated by the clinical history. It is quite possible, for example, that the pathological process in the nervous system in man may begin at the time of the first hump in "dromedary" cases. In such cases the delay in onset of paralysis would perhaps mean a more gradual spread of the destructive process in the cord as compared with the more commonly fulminating disease in artificially inoculated experimental animals. Moreover, the virus strain may play a determining role, since even in rhesus monkeys the disease is more insidious in onset when "mild" virus strains are used than when "severe" strains are involved.

The impression was obtained that in the human cases, as compared with monkeys, the motoneurons were more likely to be completely spared if they were not destroyed. This was suggested by an apparently greater proportion of normal cells in the early recovery period, but the human cases were too few in number to permit safe generalization.

Our chimpanzee material examined for comparison with the rhesus monkey was not extensive, but showed close similarity to analogous cases of rhesus poliomyelitis (compare Plate 15, figs 1 to 6, and Plate 8, figs 1 to 6).

Comparison of Poliomyelitis with Other Neurotropic Virus Diseases

Although extensive material was not available for comparing the sequence of cytopathological changes in motoneurons in various neurotropic virus diseases, several interesting similarities were found. For example, the rapidly appearing and progressing diffuse chromatolysis characteristic of early poliomyelitis is also found in rhesus monkeys infected with equine encephalomyelitis virus, and with neurotropic yellow fever virus. In all three diseases the diffuse chromatolysis of Nissl substance is the first occurrence in spinal motoneurons, and may often be unaccompanied by any inflammatory

changes in the vicinity The time course of diffuse and central chromatolytic changes observed in poliomyelitis was also found to occur in essentially similar fashion in equine encephalomyelitis and in neurotropic yellow fever, as is illustrated by the representative figures in Plate 16, figs 1 and 2, and Plate 17, figs 1 to 3

Statistical Findings A surprising finding in this study was the very high incidence of pathological change in the motor nerve cell population, indicating an almost universal dissemination of virus in the spinal motor cell population in paralysed individuals In 40 separate limb regions of 11 animals killed 2 to 5 days after onset of paralysis, the percentage of normal cells in motoneuron populations averaged between 3 and 4% Of these limb regions, 7 supplied extremities either clinically normal or showing only mild paralysis, and these showed an average of about 10% of normal motor cells in the total cell populations Five of these extremities were in animals killed on the 5th day and in which there was no possibility of progression of paralysis These findings indicate that in some cases reversible changes may affect the majority of the motoneuron population Additional strong evidence in this regard comes from the finding that whereas normal cells increase from an average proportion of 4% to 50% during the first month after the height of paralysis, destroyed cells do not increase noticeably during this period In confirmation of the latter point, moreover, necrotic cells not yet absorbed are negligible in numbers after the third day

Another important finding concerns the overall death rate of motor nerve cells in relation to the maximal degree of paralysis in the disease The average proportion of destroyed cells, we have shown, does not vary appreciably after the height of paralysis is reached (2 to 3 days after onset of paralysis) This proportion of the total motoneuron population is about 47%, and represents also the grand average of the 120 limb populations examined in this period Since, on the average, all but 4% of motoneurons are involved in the acute stage, the average proportion of injured nerve cells which are destroyed, or the average "case fatality rate", is almost 50% The range in our series of cases from the second to the fifth day varies from 12% to 91% One can therefore state that in this series as a whole there was a 50% probability that an invaded motor nerve cell would be destroyed by virus activity

The finding of a generally high incidence of infection of motoneurons obviously indicates that the disease is not necessarily arrested by failure of dissemination of virus to the entire motoneuron population, since the "exposure" of cells as shown by pathological changes is almost universal in some cases. This finding also brings up the important but difficult question of what factors produce the arrest of the disease in some motoneurons and not in others. In the present state of our knowledge of the virus-neuron reaction, an adequate consideration of the factors which may determine cell death or cell recovery is impossible, but two lines of speculation suggest themselves. First of all, it has been shown that experimentally induced changes in the metabolism of nerve cells can greatly increase their resistance to destruction by poliomyelitis virus (Howe and Bodian, '41). It is possible that variation in the normal state of the susceptible nerve cell at the time of infection may be sufficient to account for variation in the outcome of infection. On the other hand, the rapid decline of virus concentration in infected spinal cord in the acute stage (Bodian and Cumberland, '47) suggests the possibility that immune factors begin to operate early in the disease. Since the monkey motoneuron population initially consists entirely of non-immune cell individuals, arrest of the disease by antibody factors would require a very rapid mobilization of such factors locally, perhaps in the nerve cell itself. It is conceivable that variations in the rate of virus multiplication or the speed of mobilization of such immune factors might determine the outcome of cell infection. Finally, as was mentioned before, it is clear that variations in the properties of different virus strains, as well as host factors, play a role in this problem.

The high incidence of infection of motor nerve cells in paralytic cases and the fact that second paralytic attacks may occur in individuals exposed to heterologous virus strains suggest too that it is possible for a recovered nerve cell to be re-infected by heterologous virus. In several rhesus monkeys in which second paralytic attacks had occurred after an initial attack with heterologous virus several months previously, and involving severe paralysis with atrophy of at least one extremity, we found extensive acute changes in motoneurons. We have also observed two instances in which the second attack was non-paralytic, and in which widespread chromatolysis in motoneurons

gave conclusive evidence of a fresh infection due to the second inoculation. In such cases, the conclusion is inescapable that nerve cells previously infected and recovered were re-infected by heterologous virus. Presumably reinfection of cells with homologous virus is prevented by the presence of a corresponding antibody "barrier" which precludes access of virus to the cell.

Relation of Changes in Motoneurons to Paralysis and Recovery In an earlier report we have listed several possible pathological factors which may be concerned with the interference with motor function in poliomyelitis and with recovery (Bodian, '46). It was shown that interference with motor performance, especially preceding the onset of lower motor neuron weakness, could be the result of severe lesions which frequently occur in brainstem centers. Of these, the reticular formation damage is the most likely source of the spasticity which occurs in some cases, and the damage to the fastigial nuclei of the cerebellum, and to the associated vestibular nuclei, the probable source of the tremor and ataxia which are sometimes manifest. In this report, we have dealt only with the two symptoms due to lower motor neuron injury, namely decrease of muscle power, which we have referred to as paralysis, and muscle atrophy. It seems reasonably certain from the evidence now presented that the pathological and recovery changes in motoneurons alone can account for most of the paralysis and its early recovery, respectively. Other factors probably play a secondary or even negligible role, although the possibility that they play a greater role in special cases cannot be excluded from the evidence at hand. The slow, but sometimes significant, increase of power in paralysed limbs after the third month has not been studied in detail by us because of the small numbers of animals available. Since the morphological and probably functional status of the motoneuron population does not seem to change significantly after the first and second months, such recovery as occurs after this time probably is the result of slower compensatory changes in the entire neuromuscular mechanism. In a small number of monkeys studied for as long as a year after onset of paralysis, the late recovery was regularly associated with increase in size of previously atrophied muscles. Since this process is continuous and apparent for as late as 1 year, it most likely represents a gradual compensatory hypertrophy of muscle fibers with intact innervation rather than the

result of reinnervation of denervated muscle fibers by compensatory branching of surviving motor axons, as postulated by van Harreveld ('45), and by Weiss ('46)

Degree of Cell Injury in Relation to Functional State and to Virus Multiplication It is interesting to speculate on the possible relationship between the degree of cytological injury and of functional impairment. It is probably safe to assume that many neurons quite devoid of Nissl substance are irreversibly injured ("acidophilic necrosis") and non-functional, especially since their nuclei also show severe changes. At the other extreme, it appears that motoneurons showing early mild chromatolysis may still be functionally intact. In several of our pre-paralytic cases the overwhelming majority of motoneurons supplying an extremity showed mild degrees of chromatolysis in the absence of clinical signs of weakness in the extremity. Of greater interest is the suggestion of our statistical analysis that, whereas neurons showing mild chromatolysis are functional, those showing severe chromatolysis are on the borderline of function and lack of function. Many which are functionless probably recover function during the first month or two. This is not only apparent in the analysis of our paralytic cases, but explains the absence of weakness or very slight weakness in animals inoculated with "mild" strains, yet showing chromatolytic changes in over 90% of motoneurons of the limb segments. In such cases, since virus titers in infected cord may be as high as those in severely paralysed cases, it is probable that considerable virus multiplication in infected neurons need not result in cell death although the host cells undergo reversible chromatolytic changes. The difference between "mild" and "severe" strains appears to reside only in the greater necrotizing effect of the latter.

The progressive diffuse chromatolysis most likely represents an orderly process of regression of motoneuron functions, and since it is the predominant visible cytopathological change in the pre-paralytic and early paralytic period, when virus concentration in the tissue increases most rapidly (Bodian and Cumberland, '47), it may conceivably be associated intimately with the actual process of virus multiplication. The fact that the Nissl substance is known to be composed at least in part of ribonucleoprotein (Landstrom, et al, '41, Gersh and Bodian, '43) makes this speculation especially attractive.

ASSOCIATION OF PATHOLOGICAL STAGES IN MOTONEURONS WITH SPINAL VIRUS LEVELS

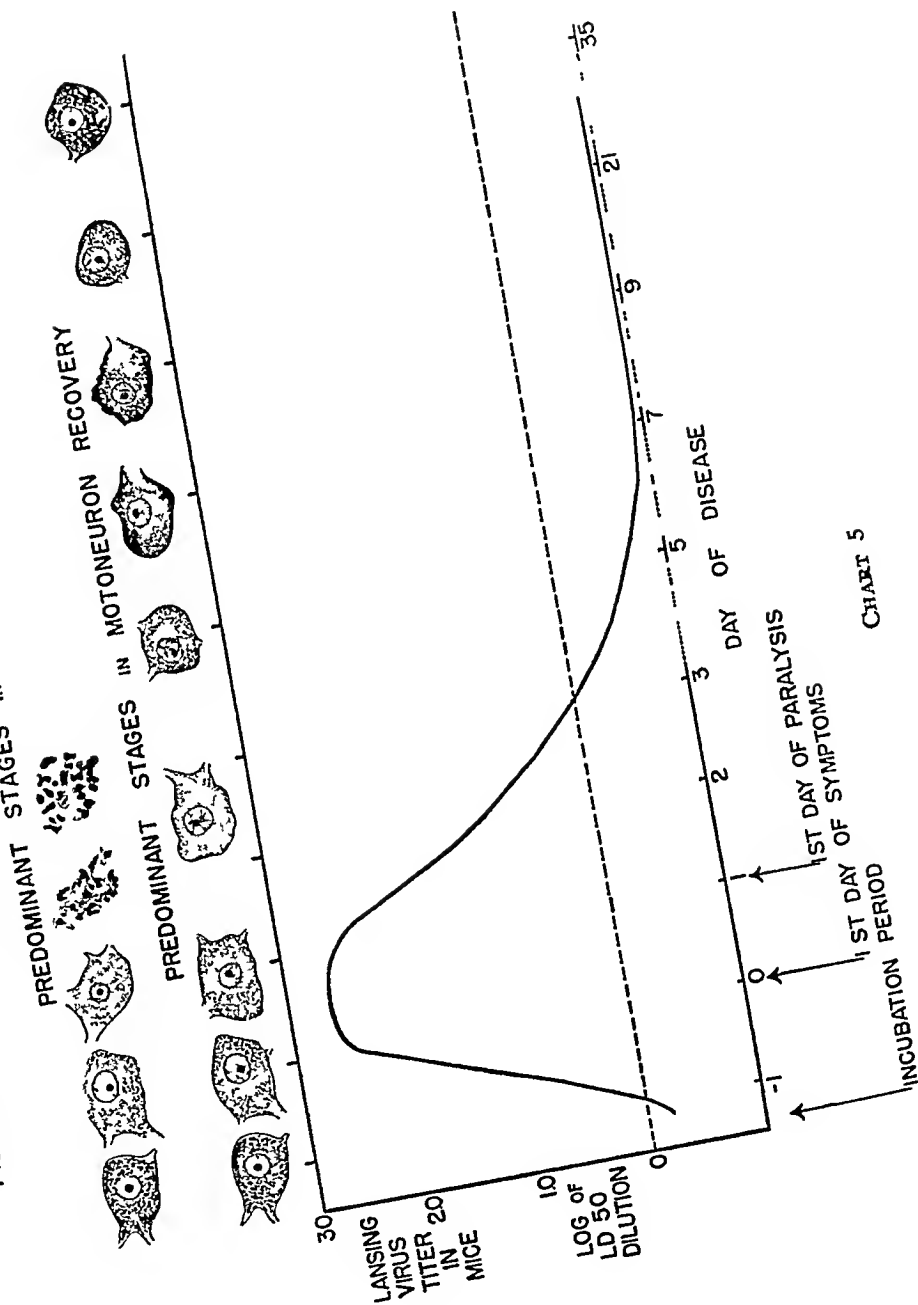


CHART 5

Nevertheless, the progressive diminution of Nissl substance may also conceivably represent the results of a gradual deterioration of cell metabolism due to the interference of virus multiplication with other cell functions. Chart 5 illustrates the time course of changes in virus concentration, as previously described (Bodian and Cumberland, '47), and the association in time of stages in the cytopathological process which dominate at each period. The fact that the greatest multiplication of virus occurs at the time when the predominant cytological change is that of early diffuse chromatolysis is suggestive. After the peak of virus concentration is achieved, stages of "central" chromatolysis appear and gradually predominate over the "diffuse" chromatolysis characteristic of the earlier period. These stages of central chromatolysis are not only associated with reduction of virus activity, but also with cessation of increase of paralysis, and then with the early recovery from paralysis.

Persistent Perivascular Lesions The inflammatory changes in acute experimental poliomyelitis have been dealt with exhaustively by Hurst ('29) and by Pette, Demme, and Kornyei ('32), and nothing new has been added by this study. It is interesting to dwell momentarily on the finding of increasing size of perivascular lymphocytic infiltrations in the subacute and convalescent stages. Such infiltrations sometimes reach massive proportions as late as the second month, often when the interstitial infiltrations no longer contain an appreciable number of these cells. The perivascular "cuffs" are negligible in size, or absent,

CHART 5 SCHEMATIC REPRESENTATION OF SEQUENCE OF CYTOPATHOLOGICAL STAGES IN MOTONEURONS IN THE COURSE OF DESTRUCTION, AND THOSE CHROMATOLYZED BUT ABLE TO RECOVER

The approximate time course of changes is shown, with a parallel curve showing the trend of rise and decline of virus concentration in the rhesus spinal cord (from Bodian and Cumberland, '47). Note that peak levels of virus concentration are attained at the time when the predominant stage of cell change in the motoneuron population is that of diffuse cytoplasmic chromatolysis.

The curve is a partly hypothetical one, showing Lansing virus activity in the rhesus spinal cord. The curve up to the third day of paralysis is based on median values of several specimens of infected monkey cord taken at each time period and titrated in mice. The decline beyond the third day of paralysis is shown in a speculative way, and is based on the decreasing probability of obtaining virus in concentrations infective by monkey passage.

in most cases after the sixth month Warburg's ('31) interpretation of such persistent lesions as a sign of smoldering activity and therefore as an indication for prolonged bed rest must be considered with much reservation First of all, no evidence that such persistent lesions are associated with virus activity can be brought forward, whereas available evidence indicates that virus activity is negligible after the third week, if it persists that long Secondly, recrudescence of the disease at such a late date has not been observed in monkeys who are always active to the limit of their capability Another interpretation of the increase of perivascular lymphocytic infiltrations in the subacute and convalescent stages is suggested by the finding that local antibodies reach a high level in infected regions of the CNS at this time, although relatively low in titer in the serum (Morgan, '47), and by the recent evidence that lymphatic tissue is concerned in antibody formation (McMaster and Hudack, '35, Ehrich and Harris, '42, Dougherty, Chase, and White, '44) It may not be too far-fetched to suppose that the reacting lymphatic tissue exhibits a continuing response in the subacute and convalescent stages to the antigenic stimulus of the acute stage, rather than being an "inflammatory" response to continued virus activity This suggested significance of the delayed lymphocytic cuffing, however, can only be considered very tentatively until experimental evidence is available

SUMMARY

1 A description is given of the course of a poliomyelitic infection in the spinal cords of rhesus monkeys killed at intervals from the pre-paralytic period to the chronic stage

2 Cytopathological changes in motor nerve cells were studied quantitatively to enable correlations with changes of virus activity on the one hand and changes in clinical motor function on the other

3 The sequence of cytopathological changes in intracellular structures is considered Those occurring in the basophilic Nissl substance of the cytoplasm appear to be valid indicators of the functional state of the neuron Mitochondria and neurofibrils were found to be morphologically intact except in necrotic cells It was found that axons degenerated between three and four days after cell body destruction, but usually not earlier than the third day This is the typical time

course of secondary degeneration, and indicates that the primary damage to motoneurons is in the cell body. Nuclear changes were found to occur only after onset of cytoplasmic chromatolysis and are described. The signs of irreversible nerve cell injury are discussed.

4 The general features of the cellular pathological picture are described for stages of the disease from the preparalytic to the convalescent periods.

5 The cytopathological changes in motoneurons were grouped in presumptive stages of regression and recovery and studied with respect to the changing proportions of normal cells, abnormal stages, and destroyed cells in the motoneuron populations supplying single limbs. Limbs of all grades of paralysis were studied, with the average of the order of "moderately severe" paralysis.

6 In paralytic animals it is shown that the process of nerve cell destruction rapidly reaches a peak within the first few days after onset, and shortly thereafter is negligible in relation to the total cell population.

7 In paralytic animals, also, it is shown that as a rule over 90% of all motoneurons in the limb populations are infected during the first few days, as is indicated by abnormal cytopathological changes in the cells. This is true of limbs with mild or transient paralysis as well as those severely paralysed.

8 These changes first involve the Nissl substance, and are progressive in an orderly fashion until changes set in which seriously involve nuclear structure. Short of such irreversible changes, the cytopathological changes in Nissl substance are slowly reversed during the course of 4 to 6 weeks, or less, so that after this time most remaining motoneurons are normal in appearance.

9 Statistical evidence from populations of motoneurons studied during acute and recovery periods indicates conclusively that a large proportion of motoneurons injured to the extent of severe loss of Nissl substance recover their normal appearance. The proportion is greatest in limbs with at least moderate paralysis, but is high in all but the most severely paralysed limbs, in which little function ever returns. In not a single paralytic case between the second and seventh days of paralysis was there found more than 31% of normal cells, even in cell populations supplying limbs with minimal or no apparent paralysis.

10 The relation between damage and recovery of the motor nerve cell population to motor paralysis and recovery is considered. It appears that the degree of motoneuron destruction alone in a limb motoneuron population can account for most of the weakness observed in the corresponding limb in the period between 21 days and 3 months after onset of paralysis. On the average, limbs graded as normal in this period had lost about $\frac{1}{3}$ of their motoneurons, and those graded as completely paralysed still retained about 10% of the motoneuron population.

11 The degree of weakness in the acute stage was greater than could be accounted for by nerve cell destruction alone, but evidence suggests that the discrepancy may be related to the absence of function in severely chromatolysed motoneurons which apparently later recover their function. There is evidence that suggests that neurons showing mild degrees of chromatolysis are still functional in that state.

12 The distribution of neuron lesions was almost universal in the nerve cell populations of all four limbs in paralysed animals and in most animals with non-paralytic poliomyelitis. This was also true of animals infected with "mild" strains and showing very slight weakness. No consistent pattern of cell invasion was found, but there was a suggestion that cells on the periphery of the anterior columns of limb enlargements had a better chance for survival. Some of the factors which may be involved in determining the pattern of paralysis are discussed.

13 Evidence is presented which indicates that highest concentrations of virus are achieved in the anterior horn when the predominant pathological change in the motoneuron population is that of diffuse cytoplasmic chromatolysis of Nissl substance.

14 Perivascular lymphatic accumulations, occasionally with germinal centers, persist for months after acute neuronal changes, acute inflammatory changes, or evidence of virus activity can be found. It is suggested that such persisting lymphatic tissue may be associated with the occurrence of local antibody.

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EXPLANATION OF ILLUSTRATIONS

Except where otherwise noted, sections were prepared at 15 micra and stained with gallocyanin

(Photographs were prepared by Mr Chester F Reather)

PLATE 1 REGRESSIVE STAGES IN SPINAL CORD MOTONEURONS IN
POLIOMYELITIS RHESUS B12, FIRST DAY OF PARALYSIS, $\times 480$

FIG 1 *Normal anterior horn cell* Note massive Nissl bodies in cytoplasm, central position of nucleus, large nucleolus, and dispersed chromatin

FIGS 2, 3, 4 Early regressive stages Note diffuse decrease in size of Nissl bodies (chromatolysis), and nucleus essentially normal

FIG 5 Severe diffuse chromatolysis, with only a few small masses of Nissl substance in cell periphery Note clumping of chromatin in nucleus

FIG 6 Complete dissolution of Nissl bodies in cytoplasm, which is slightly basophilic in staining Nucleus slightly shrunken and containing a small eosinophilic inclusion body

FIG 7 Cell similar to that in fig 6, with further shrinkage of nucleus Note infiltrating "polyblasts" surrounding the nerve cell

FIG 8 Completely chromatolytic cell with severe diffuse basophilia of cytoplasm and of shrunken, distorted nucleus

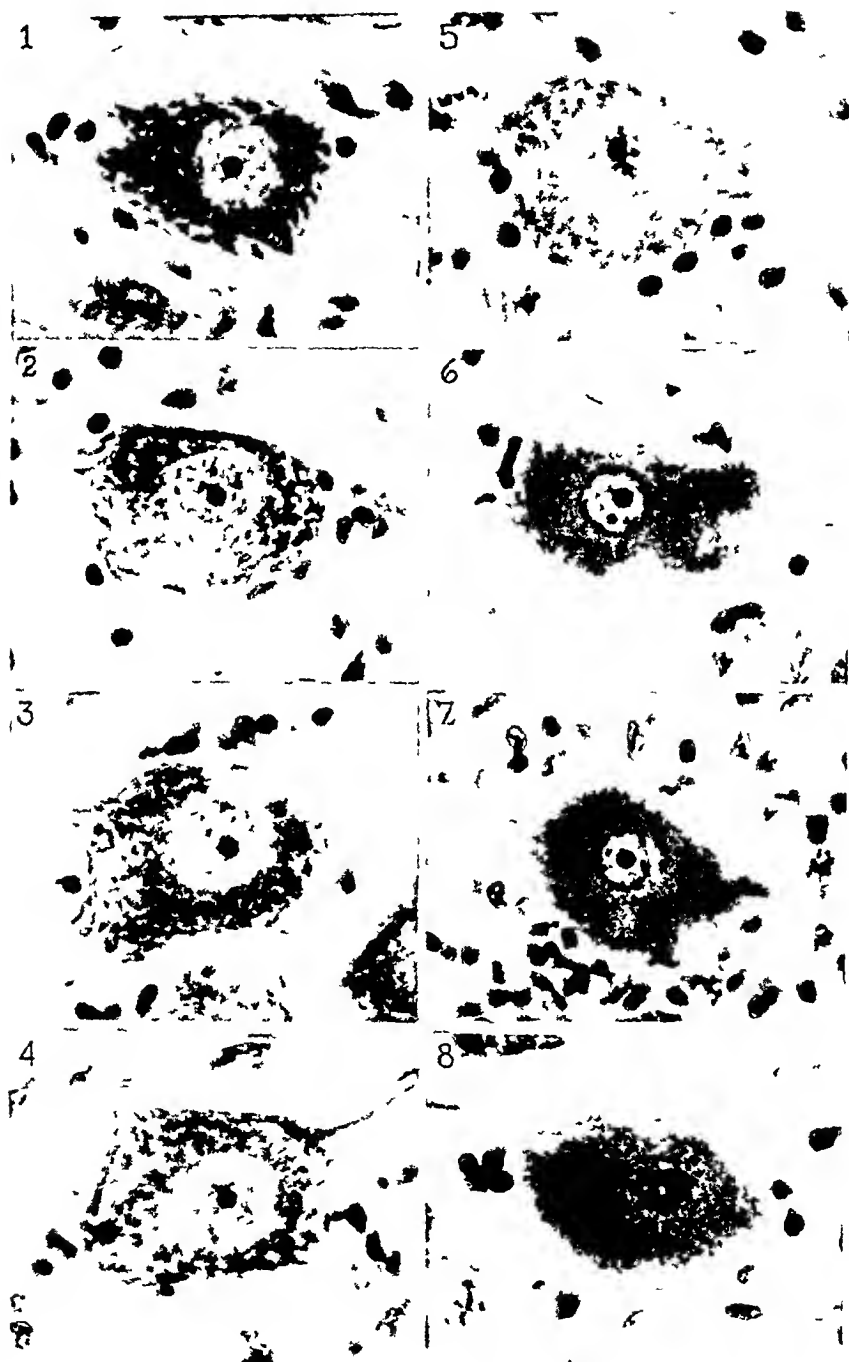


PLATE 2 Stages of degeneration of motoneurons during first day of paralysis, showing destruction by phagocytosis (fig 1 to 4), by cytolysis (figs 5 and 6), and by vacuolation with absorption (figs 7 and 8) Figs 1, 2, 3, 5, 7, and 8 from rhesus B12 Figs 4 and 6 from rhesus A696 $\times 480$

FIG 1 Intensely hyperchromatic cytoplasm and shriveled nucleus Such basophilic cells are more commonly phagocytized than those which become acidophilic Note blood capillary enclosed by cytoplasm of cell

FIG 2 Shrunk, hyperchromatic cell, with granular and vacuolated cytoplasm and pyknotic nucleus Beginning neuronophagia by surrounding leucocytes and macrophages

FIG 3 Active neuronophagia of necrotic cell

FIG 4 Macrophages and glia cells at site of a completely phagocytized cell

FIG 5 Complete chromatolysis, with no trace of basophilia in cytoplasm Such cells appear to be on the verge of "acidophilic necrosis", or the cytolysis and vacuolation shown in figs 6 to 8

FIG 6 Cytolysis of cell with shrunken nucleus showing a beaded, basophilic border After complete cytolysis, a punched out, fluid-filled cavity is left ("falling out"), which is reduced in a short time by shrinkage and the infiltration of macrophages, etc

FIGS 7 AND 8 Cells in final stages of vacuolation, with surrounding and invading phagocytic cells

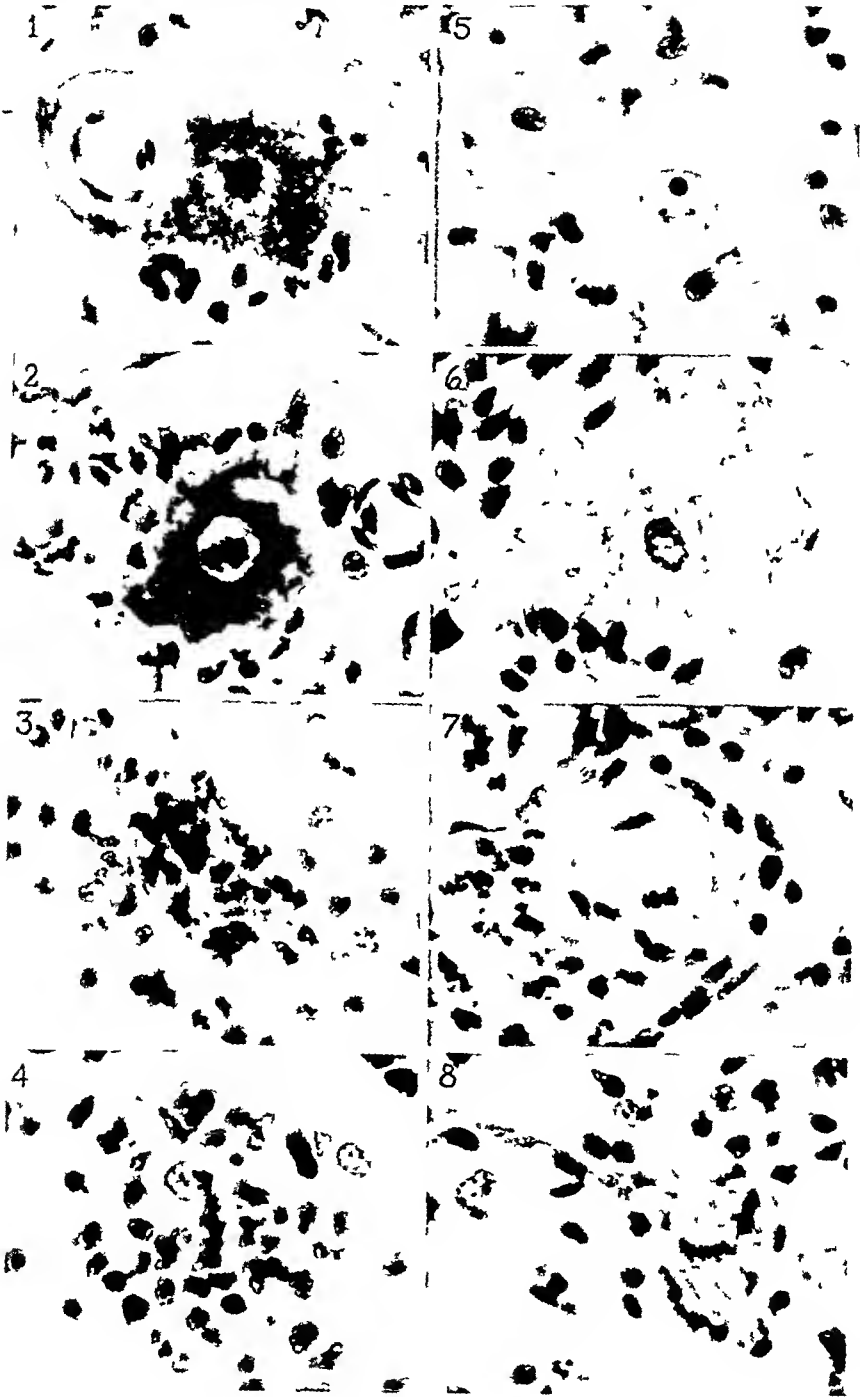


PLATE 3 DEGENERATION OF MITOCHONDRIA IN POLIOMYELITIS

FIG 1 Anterior horn cell in stage of severe poliomyelitic chromatolysis, with eccentric nucleus (n) containing several inclusion bodies. Note numerous granular and small rod-like mitochondria in cytoplasm (c). The pale larger masses at the edges of the cytoplasm are remaining Nissl bodies. Rhesus B916, first day of paralysis. Regaud, Iron-haematoxylin, 7μ , $\times 1300$.

FIG 2 Anterior horn cell in same section as fig 1, showing necrotic changes in nucleus (n) and cytoplasm (vacuolation, granular degeneration). No mitochondria or Nissl bodies visible in cytoplasm (c).

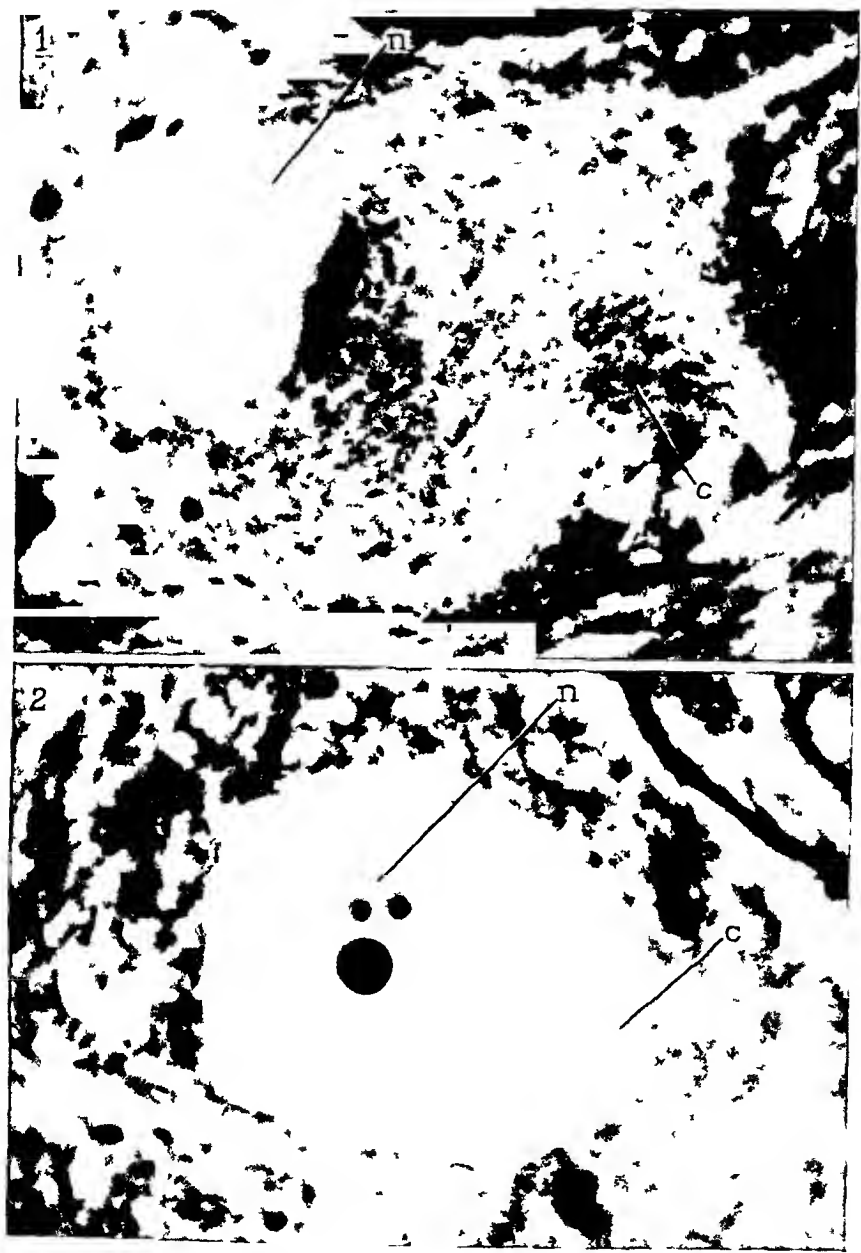


PLATE 4 DEGENERATION OF MOTOR NERVE FIBERS IN POLIOMYELITIS
PROTARGOL STAIN, 15 μ , \times 560

FIG 1 Normal anterior spinal root of rhesus monkey, showing large and small axons of fairly uniform caliber Rhesus C888

FIG 2 Emerging anterior root fiber in white matter of spinal cord of rhesus monkey on third day of paralysis Note swelling and vacuolation of axon (ax) This represents the earliest degenerative change seen in our series Rhesus C947

FIG 3 Sixth lumbar anterior spinal root of monkey with complete paralysis of left leg, showing severe degenerative changes of almost all axons in the root Fourth day of paralysis Rhesus C944

FIG 4 Sixth lumbar anterior spinal root of monkey with complete paralysis of left leg, showing complete degeneration and absorption of all axons, with replacement by neurilemmal and fibrous tissue, 57 days after onset of paralysis Rhesus C437

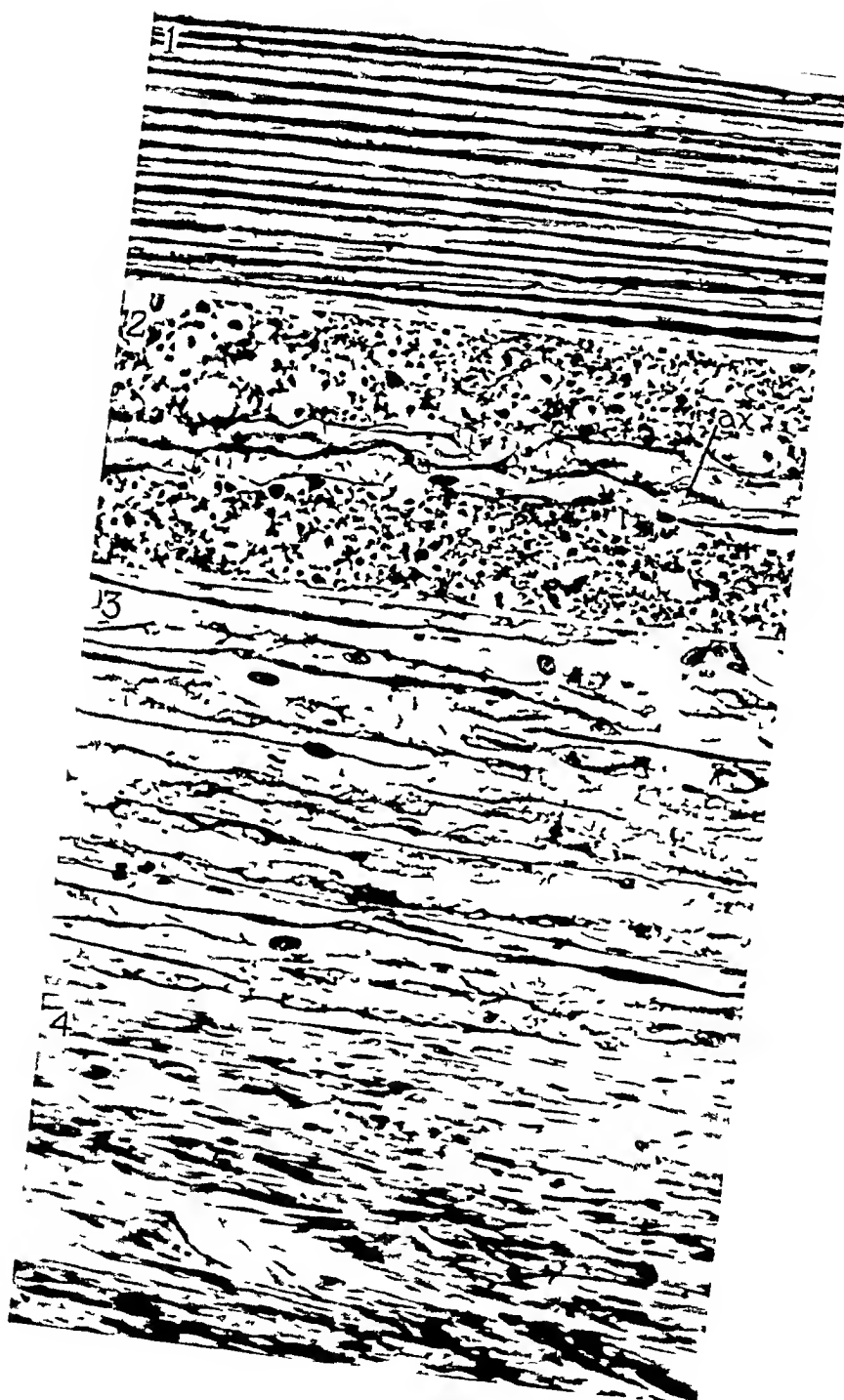


PLATE 5 Samples of the infrequently occurring motor nerve cells which persist after the acute period, although they show changes of a presumably irreversible nature ("necrotic")

FIGS 1 to 4, chimpanzee A434, 7 days after onset of paralysis, Fig 5, rhesus B32, 9 days, Fig 6, rhesus B35, 21 days $\times 480$

FIG 1 Motoneuron with eosinophilic, highly refractile cytoplasm, with shrunken and distorted cell and nuclear membranes

FIGS 2 AND 3 Similar cells, but with strongly basophilic nuclei

FIG 4 Markedly shrunken cell, with strongly basophilic masses in nucleus and cytoplasm, surrounded by macrophages Normal internal structure is not visible

FIG 5 Cell with distended nucleus, and glassy, eosinophilic cytoplasm, probably irreversibly damaged

FIG 6 Motoneuron with glassy cytoplasm, and rim of basophilic substance, showing nucleus apparently verging on extrusion from cytoplasm (See also Plate 10, fig 1)

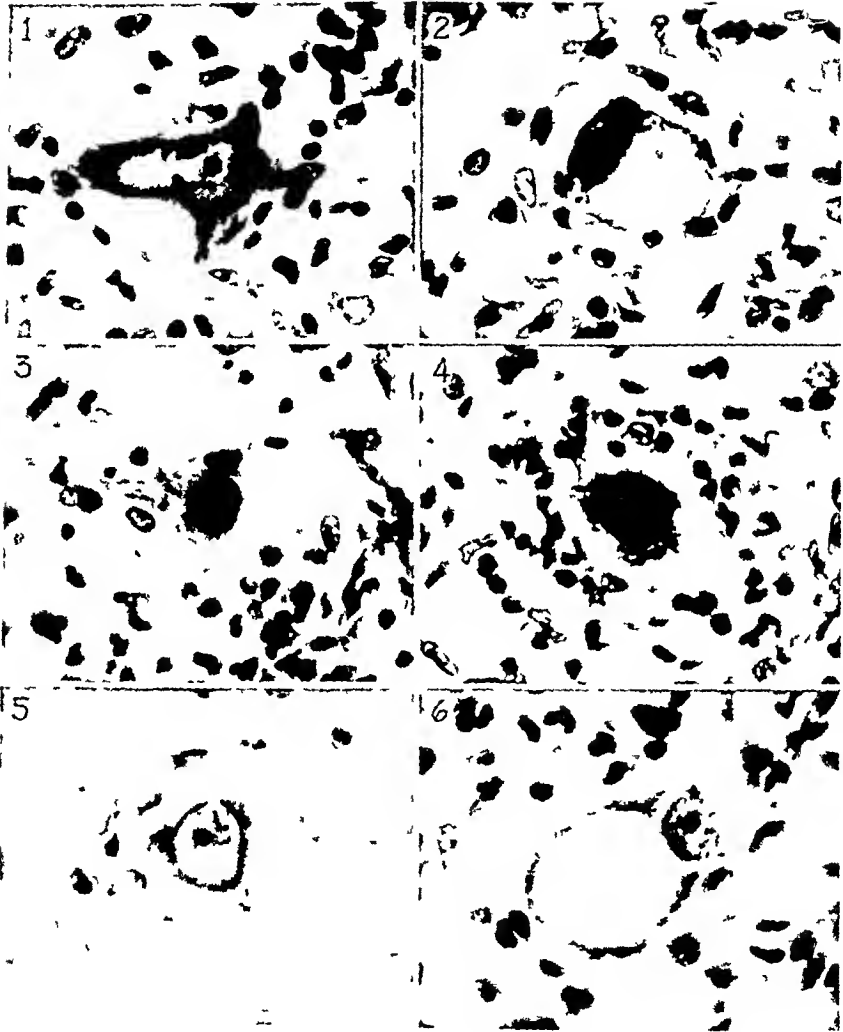


PLATE 6 EARLY PREPARALYTIC POLIOMYELITIS RHESUS A935

FIG 1 Section of cord at fourth lumbar level, showing characteristic fully developed inflammatory lesions cc—central canal pr—perivascular infiltration around vessels in anterior fissure $\times 60$

FIG 2 Motoneurons also shown in box in fig 1, at higher magnification Note early diffuse chromatolysis $\times 250$

FIGS 3-5 Sections at distance of $1\frac{1}{2}$ mm from section shown in fig 1 These sections, and adjacent ones, had no infiltrative cells in anterior horns Only the section shown in fig 5 had any infiltration in the entire section, and this was around the anterior spinal vessels The diffuse chromatolysis seen in the cells at the left was seen in these cells alone in their respective sections, and in all three cases was in the ventrolateral cell column $\times 250$

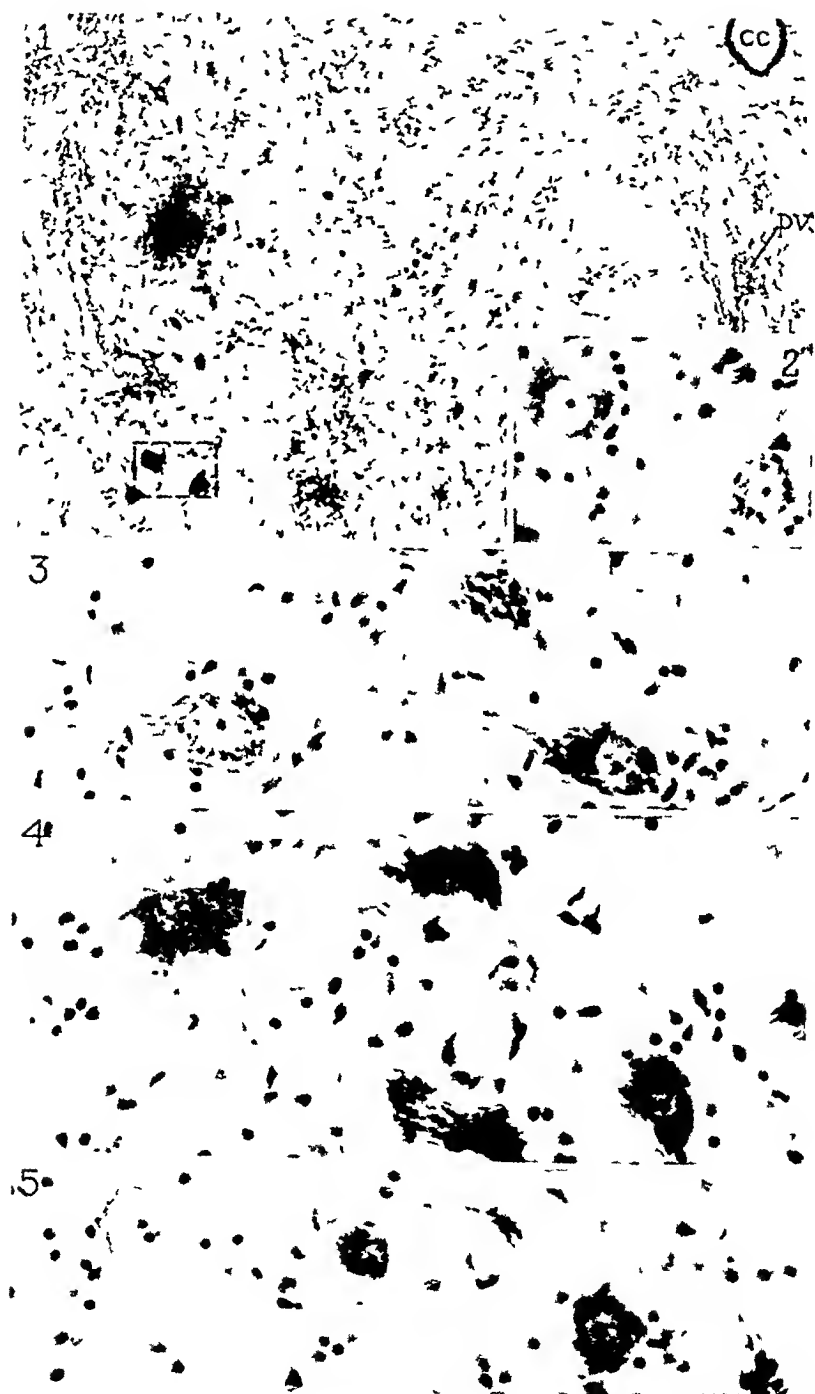


PLATE 7 Rhesus B338 Fifth day after onset of paralysis Series of cells showing the transition from regressive changes (diffuse chromatolysis) to recovery stages (central chromatolysis) $\times 480$

FIGS 1 AND 2 Motoneurons showing the diffuse cytoplasmic chromatolysis characteristic of the acute stage

FIGS 3 AND 4 Motoneurons showing a tendency for massing of Nissl substance near cell and nuclear membranes, and diffuse cytoplasmic basophilia Such cells are apparently transitional between those of figs 1 and 2, and 5 and 6

FIGS 5 AND 6 Motoneurons showing central chromatolysis, with well-stained Nissl bodies and absence of diffuse basophilia Such cells are characteristic of the entire recovery period

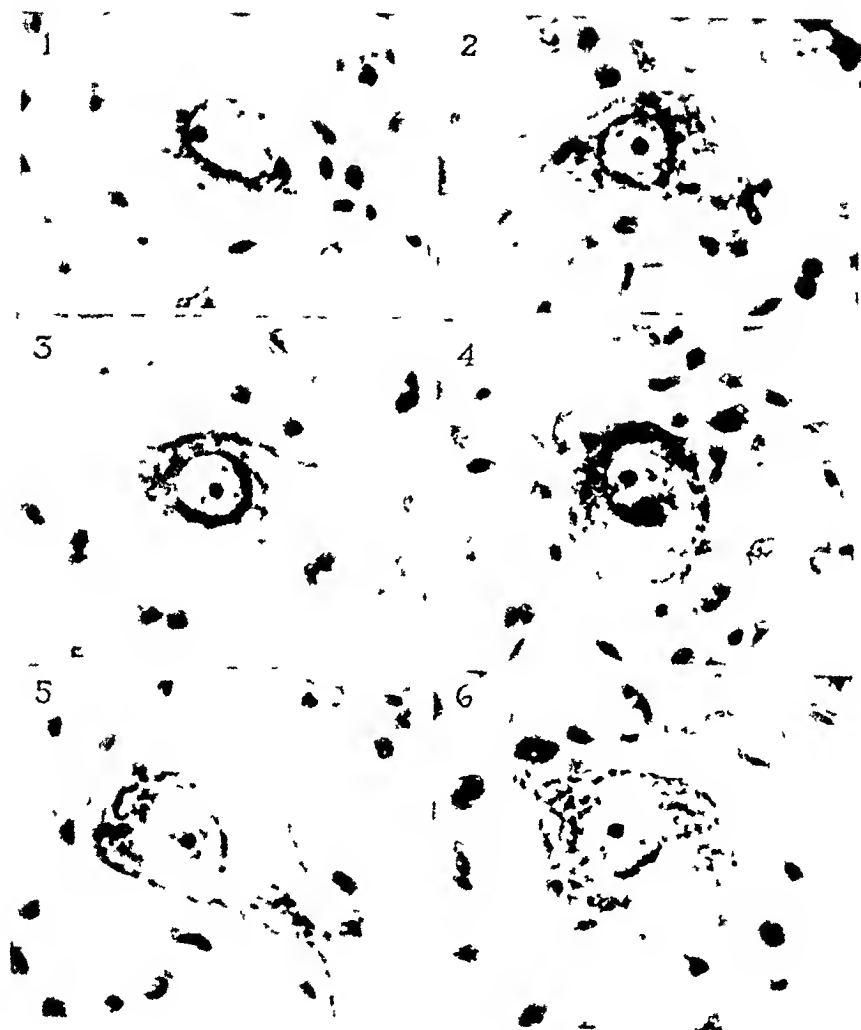


PLATE 8 RHESUS B32 NINTH DAY AFTER ONSET OF PARALYSIS $\times 480$

FIG 1 Severe central chromatolysis, with normal-appearing nucleus, and accumulation of heavy masses of Nissl substance near cell membrane. Regeneration of Nissl substance may or may not occur near the nuclear membrane.

FIGS 2 TO 5 Similar cells but with small Nissl bodies in central area. This appearance suggests regeneration of Nissl bodies from the periphery inwards, with the area around the axon hillock (ah) last to show recovery (figs 2, 4, and 5).

FIG 6 Motoneuron of essentially normal appearance, except for presence of acidophilic inclusion body (b) in nucleus. In the acute stage such inclusion bodies are seen only in cells with severe chromatolysis, suggesting almost complete recovery of such a cell.

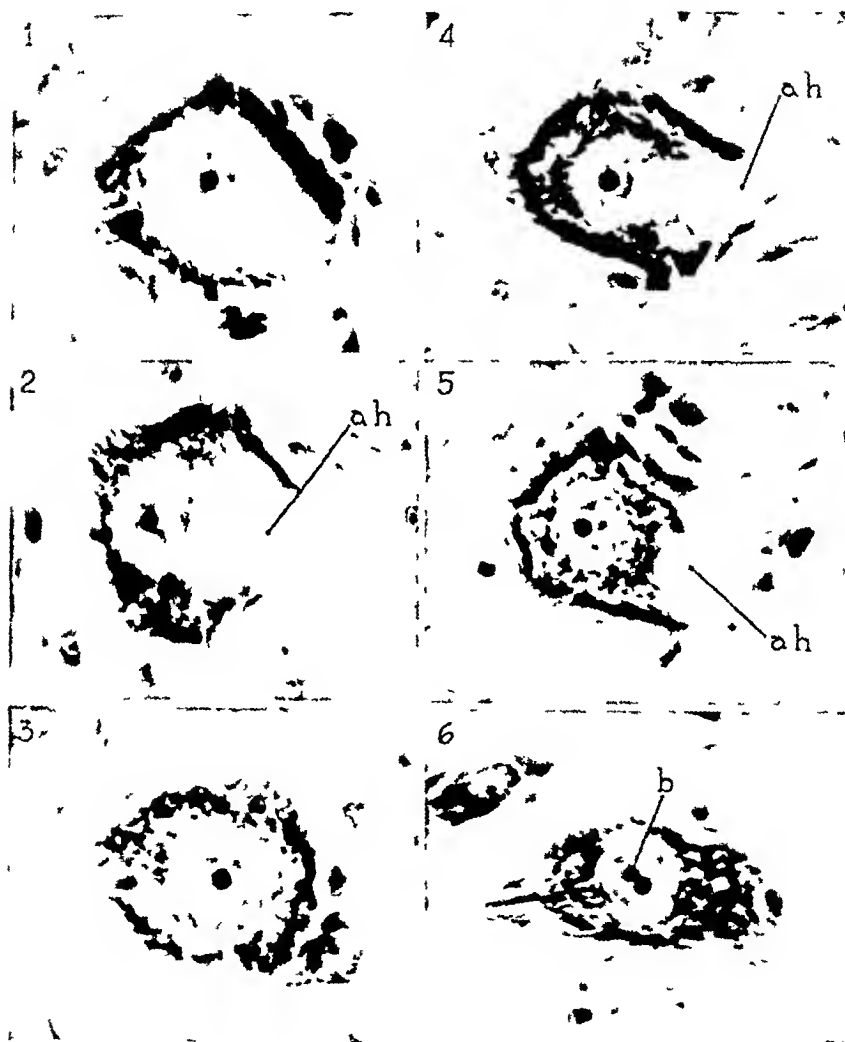


PLATE 9 INCOMPLETELY RECOVERED MOTONEURONS INFREQUENTLY
SEEN AFTER THE THIRD WEEK $\times 480$

FIGS 1 AND 2 Rhesus B35, 21 days after onset of paralysis Central chromatolysis similar to that seen in Plate 8, with acidophilic inclusion body (b) in fig 2

FIGS 3 TO 6 Rhesus B190, 35 days after onset of paralysis Motoneurons in late stages of recovery, but showing various signs of previous injury, fig 3, incomplete recovery of Nissl bodies in central area, fig 4, normal cell, except for acidophilic intranuclear inclusion (b), fig 5, cell with almost recovered Nissl bodies, but with bizarre basophilic intranuclear inclusion body (a), representing agglomerated nucleolus and surrounding chromatin, fig 6, otherwise normal cell with similar intranuclear formation

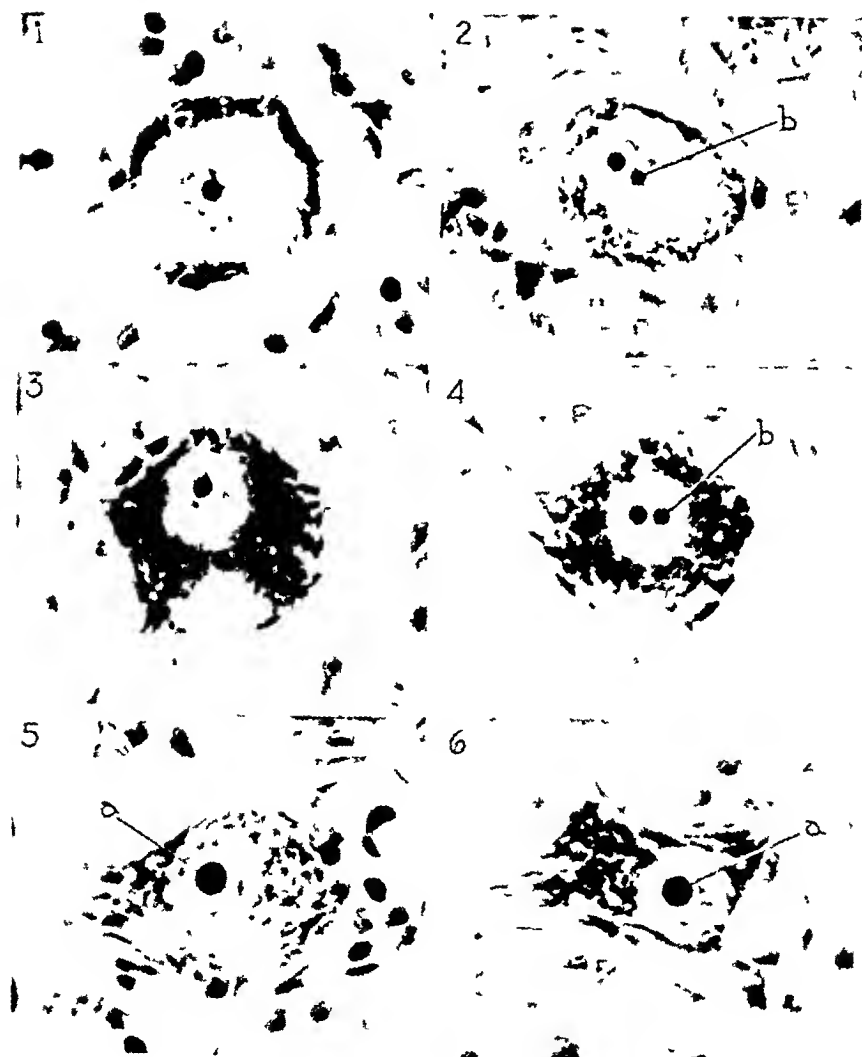


PLATE 10 Rhesus B935, 7 days after onset of paralysis, showing the changing character of reacting non-neuronal cells after the acute stage Regaud, haematoxylin-eosin-azure II, 7 micra, $\times 560$

FIG 1 Note absence of polymorphonuclear leucocytes, "rod" cells, or of the irregular "polyblasts" of the acute stage Spindle-shaped cells (h), probably histiocytes or microglia, dominate the scene, which also contains glia cells, and a few stray lymphocytes The moribund motoneuron in the center appears to be undergoing extrusion of its nucleus (n)

FIG 2 Same section as fig 1 Note the mixed character of cells in the perivascular zone In this zone in the acute stage the cells are largely lymphocytic in type Histiocytes and macrophages are numerous, as well as lymphocytes and a few cells of the "stem-cell" type, with cytoplasmic basophilia Some of the macrophages contain engulfed lymphocytes at this stage (m) The neuron at upper left is quite abnormal in appearance and contains a basophilic intranuclear inclusion of the type shown in Plate 9, figs 5 and 6



PLATE 11 Rhesus B190, 35 days after onset of paralysis, showing the second stage of lymphocytic perivascular infiltration in the early convalescent period, when lymphocytes are largely absent from the rest of the tissue

FIG 1 Massive perivascular lymphocytic infiltrations, containing "germinal" centers (c) $\times 65$

FIG 2 Same at higher magnification showing area at lower left "Germinal" center contains stem cells and mitotic figures (m) $\times 270$

1



2



PLATE 12 Normal rhesus lumbar cord, to show the sharp borders of the area containing the motoneurons of this segment of spinal cord $\times 20$



PLATE 13 The principal morphological stages of injured and recovering motoneurons, during the first week of the disease $\times 250$

FIG 1 Rhesus B12, preparalytic period Note the diffuse character of chromatolysis in the three motoneurons below The one at upper right is essentially normal

FIG 2 Rhesus A794, third day after onset of paralysis Transitional stage, with motoneurons showing "new" granular Nissl bodies, and accumulation of Nissl substance near cell and nuclear membranes

FIG 3 Rhesus B338, fifth day after onset of paralysis Early stage of central chromatolysis, with definite membrane accumulation of Nissl substance, and pale central zone of cytoplasm

FIG 4 Rhesus B935, seventh day after onset of paralysis Typical central chromatolysis with "filling-in" of central cytoplasmic area by "new" Nissl bodies

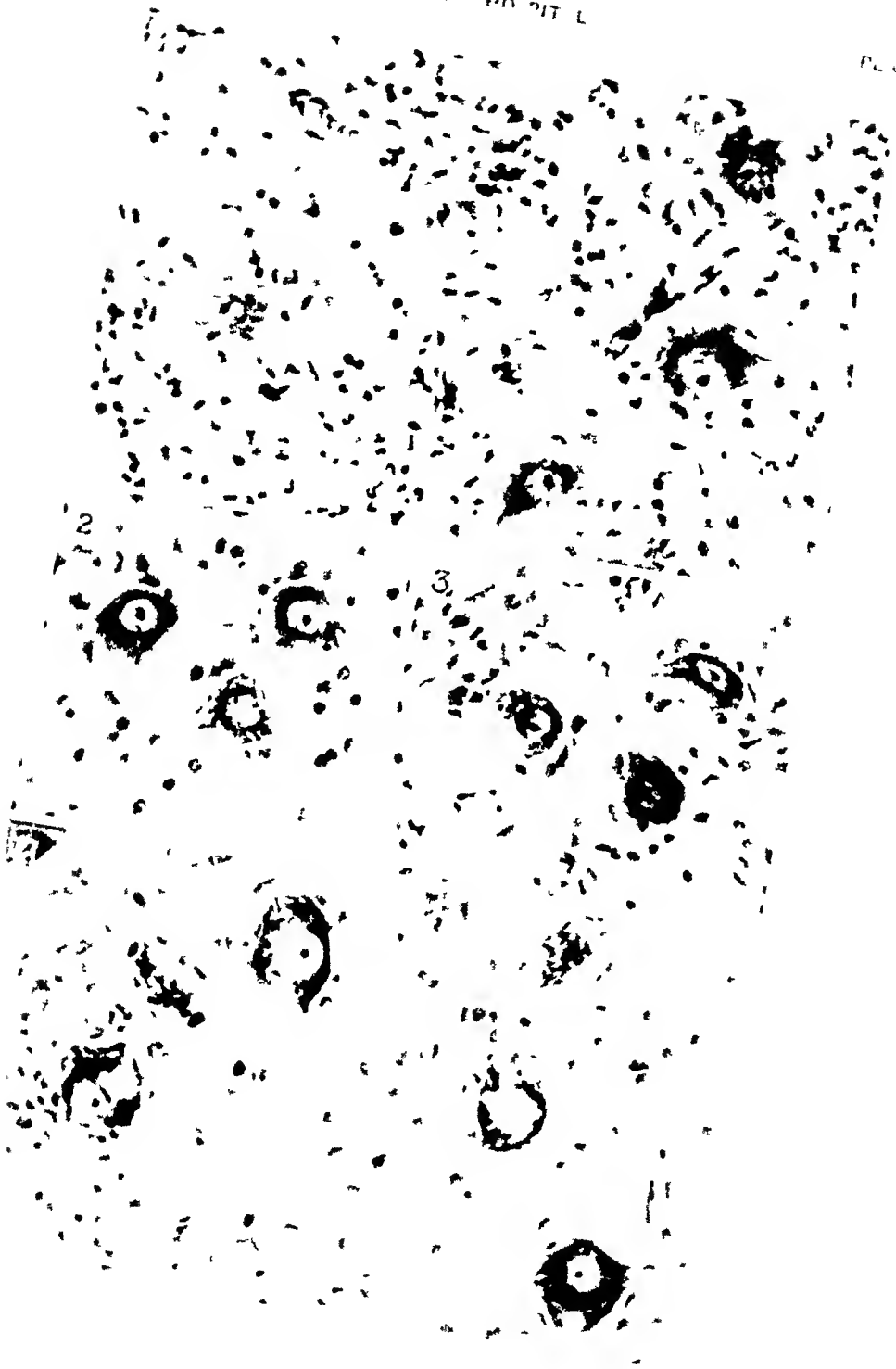


PLATE 14 Sections through lumbar cord of rhesus C59, inoculated with mild Frederick strain, with transient paralysis after 17 days, and killed 4 days later Described on page 58

FIG 1 Right anterior horn Note that all motoneurons show chromatolysis, although there is little inflammatory reaction $\times 125$

FIG 2 Left anterior horn, same section, showing neuron loss, as well as chromatolysis of remaining cells $\times 80$

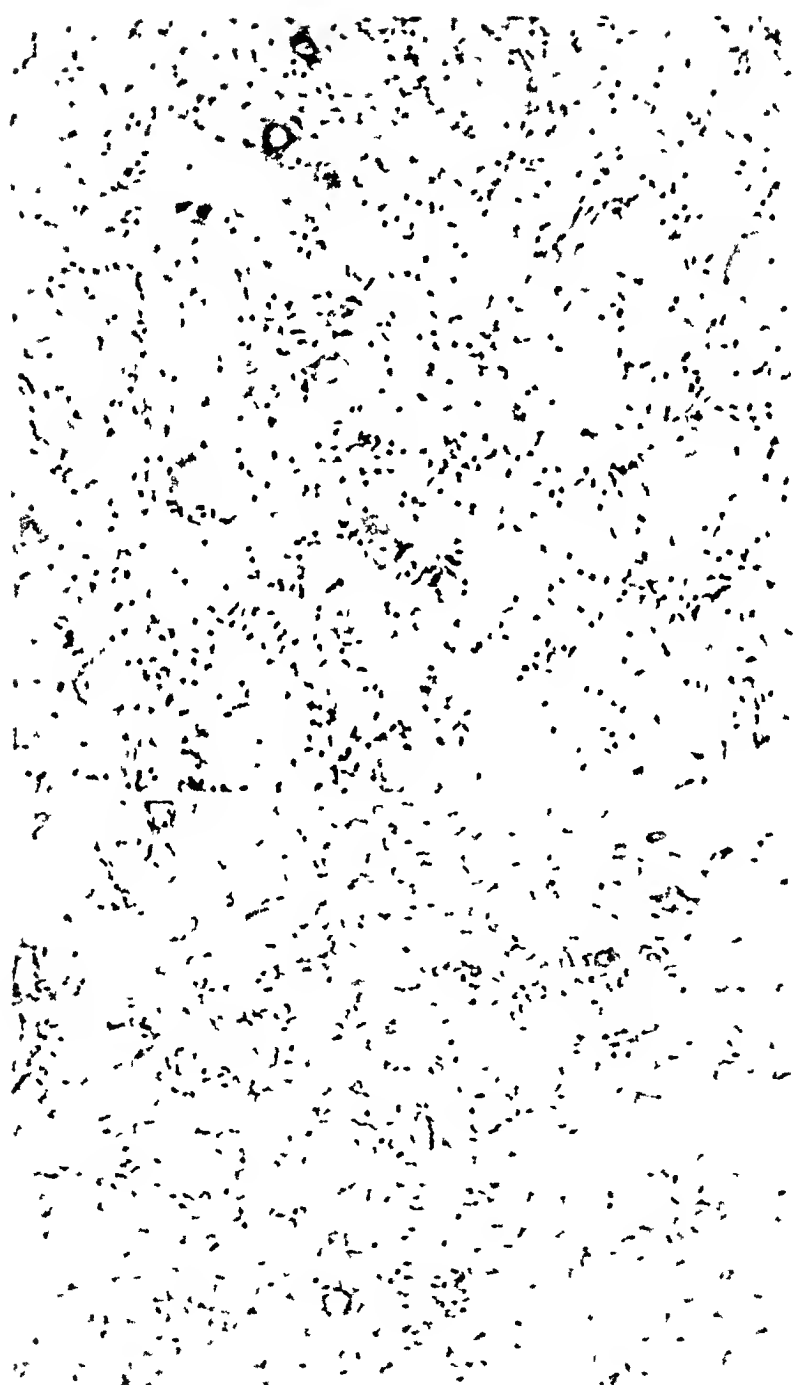


PLATE 15 CHIMPANZEE A434, SEVENTH DAY AFTER ONSET OF PARALYSIS

FIGS 1 TO 6 show a group of cells arranged in the sequence of increasing recovery from the chromatolysis of the acute stage. Note the similarity to cells of rhesus monkey B32, shown in Plate 8. $\times 480$

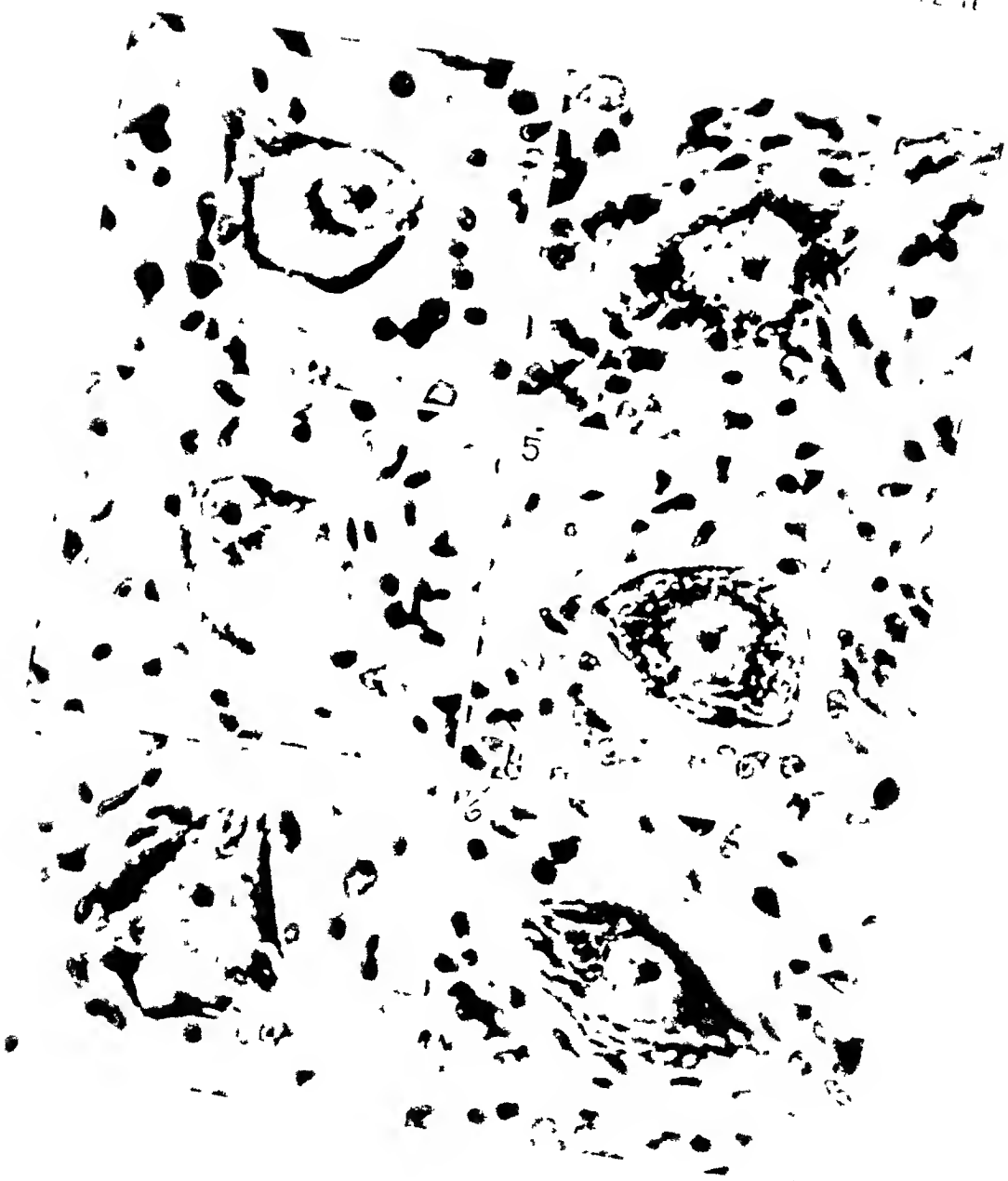


PLATE 16 Rhesus C479, showing the acute stage of non-paralytic Western equine encephalomyelitis in the cervical spinal cord $\times 270$

FIG 1 Group of anterior horn cells showing either normal appearance or severe diffuse cytoplasmic chromatolysis. Small acidophilic intranuclear inclusions are visible in the chromatolytic cells.

FIG 2 Neighboring section, showing characteristic inflammatory focus, with the same cell types seen in poliomyelitis. A single necrotic motoneuron is seen (∇), but most motoneurons show no changes, in contrast with poliomyelitis cases.

14.



PLATE 17 Three stages showing the injury and recovery of motoneurons following non-paralytic infection with neurotropic yellow fever virus (17D strain) Toluidin blue, $\times 250$

FIG 1 Rhesus C481, two days after onset of disease Note the diffuse chromatolysis of the two lower motoneurons, in the absence of inflammatory reaction Nuclei are normal

FIG 2 Rhesus C480, five days after onset of disease Note the beginning membrane accumulation of Nissl bodies in motoneuron below and at upper right Nerve cell above in center shows intense basophilia of nucleus and cytoplasm, although morphologically almost intact Such cells were not frequent in occurrence

FIG 3 Rhesus C482, eleven days after onset of disease Two large motoneurons at right show typical central chromatolysis, similar to that seen in poliomyelitis at this period in the disease Other motoneurons in the section resemble most motoneurons in the spinal cord in being normal in appearance



PROCEEDINGS OF THE MEETING OF THE JOHNS HOPKINS MEDICAL SOCIETY

HELD IN HURD MEMORIAL HALL, APRIL 12, 1948

ELECTION OF OFFICERS FOR THE COMING YEAR

Dr A McGehee Harvey was elected president and Dr Richard H Follis was elected secretary-treasurer for the year 1948-49

THE RELATIONSHIP BETWEEN INFLUENZA AND ACUTE BACTERIAL PNEUMONIA

Conflicting Interpretations of Laboratory and Epidemiological Evidence DR ALEX-
ANDER D LANGMUIR (Department of Epidemiology, Johns Hopkins Uni-
versity School of Hygiene and Public Health)

During the 1928 pandemic of influenza, bacterial infections of the lung were known to have been unusually frequent and to have contributed substantially to the excessive mortality that was recorded. During the eighteen epidemics of mild influenza that have occurred in the United States since the pandemic, the existence of a definite relation between the virus infection and a complicating acute bacterial pneumonia has been less apparent.

Although each of these eighteen epidemics has been accompanied by a measurable excess mortality from pneumonia, such statistical evidence does not determine whether these excess pneumonia deaths resulted from bacterial invasion of the lungs or merely from extensive virus infections. Laboratory studies have not clarified the question completely. The rarity of bacterial complications during localized outbreaks of influenza A and B has been emphasized on repeated occasions. Studies of patients with acute bacterial pneumonia have yielded variable results. Although relation between influenza and acute staphylococcal pneumonia is now well established, evidence of a similar relation with pneumococcal pneumonia is very limited.

An epidemiological study of the records of the pneumonia service at Bellevue Hospital, New York, is now in progress in collaboration with Dr Norman Plummer. These records comprise case histories of patients with pneumococcal pneumonia admitted between October 1920 and June 1932. During this period, six epidemics of influenza occurred in New York City. During four of these epidemics, the weekly incidence of admissions for acute bacterial pneumonia increased sharply by 50 to 100 per cent or more. In the other two epidemics slight but less definite increases were observed.

This epidemiological evidence indicates that during certain epidemics of influenza as much as 50 per cent or more of the cases of pneumococcal pneumonia may represent concurrent secondary bacterial complications of primary virus infections.

Elucidation of this mechanism is one logical approach toward a better understanding of pandemic influenza

Demonstration of Influenza Virus Complicating Pneumococcal Pneumonia, 1946-1947

DR THOMAS G WARD (Department of Bacteriology, Johns Hopkins University School of Hygiene and Public Health)

Evidence presented herein suggests that influenza virus is one of the causative agents in cases of pneumonia previously considered primarily bacterial in origin. Sputum was obtained from 69 cases of bacterial pneumonia and studied for influenza virus by the chick embryo technique. Of 33 cases occurring during non-influenza periods one yielded an influenza B virus. Of 36 cases occurring during the period when influenza A was prevalent in Baltimore, 13 yielded influenza virus which were serologically similar to influenza A strains isolated from clinical cases of influenza occurring at the same time. Acute and convalescent blood specimens were secured on 53 of these same cases, and a third specimen was obtained about five months later on 25. Four cases showed evidence of positive hemagglutination-inhibition antibody response but failed to yield virus. Thus a total of 17 of 36 cases (47%) of bacterial pneumonia occurring during an influenza A epidemic gave evidence, by virus isolation or serologic techniques, of the presence of influenza virus associated with bacterial pneumonia.

In addition, a total of 87 specimens of lung secured at autopsy were studied. Two of nine cases whose death was attributed primarily to pneumonia yielded influenza virus. Two of 28 cases whose primary cause of death was complicated by pneumonia also yielded influenza virus. No virus was secured from the remaining 50 cases which presented no evidence of pneumonia. Twenty-eight of these specimens were secured during the influenza epidemic in Baltimore, and none of these yielded virus.

To summarize, these studies demonstrate that influenza virus probably plays an etiological role in the production of bacterial pneumonia in man.

The Clinical Features of Pneumococcal Pneumonia Complicated by Influenza Virus Infection DR THOMAS E VAN METRE (Department of Medicine, The Johns Hopkins Hospital)

The preceding papers showed that influenza might coexist with bacterial pneumonia. Does influenza modify the course of concurrent bacterial pneumonia? The clinical records of 49 non-fatal cases of pneumococcus pneumonia that were studied for the presence of influenza by Dr Ward have been analyzed. In 18 of these cases influenza was present by the criteria previously discussed. The 18 cases with influenza showed no significant difference from the 31 cases without influenza beyond a higher incidence of symptoms of influenza preceding or concurrent with the onset of pneumonia. These findings concur with those of Finland on non-fatal cases of pneumococcal pneumonia complicated by influenza.

The clinical and autopsy records on two fatal cases of pneumonia in which influenza virus was isolated from the lungs were presented. The first patient was a 7-month-old, white, male infant who died in four days of a progressive tracheo-broncho-pneumonitis. Type XI pneumococcus was present in the sputum, but there was no response to penicillin and sulfadiazine. At autopsy, a necrotising tracheitis and an interstitial bronchitis and pneumonitis were found, the picture of pure virus infection. No bacteria could be stained or cultured. Influenza B virus was isolated from the lung. This case was interpreted as one in which death was due to influenza virus infection.

The second patient was a 25 year old negress who died in 32 hours of a lobar pneumonia involving RML, RLL, LLL, LUL, despite the fact that penicillin and sulfadiazine were started 12 hours after her first symptom. Type II pneumococcus was demonstrated in lung and nasopharynx. Influenza A virus was isolated from the lungs. At autopsy a typical early pneumococcal pneumonia involving all but the lung apices was found. A very few gram-positive, encapsulated diplococci could be stained in the alveoli. There were no lesions typical of virus infection. Moderate mitral stenosis was also found. The presence of influenza A suggested that the virus might have been responsible for the fulminating course and the absence of response to adequate specific therapy for the pneumococcus.

Dr William B Vandegrift What was shown by bacterial stains of the lung in the second case?

Dr Thomas E Van Metre Bacterial stains showed almost no evidence of bacteria. Only three or four gram-positive diplococci could be found in an entire section. Culture of the lungs revealed a few colonies of type II pneumococci.

Dr Vandegrift In the second case were the lung lesions typical of virus or pneumococcal pneumonia?

Dr Van Metre In the second case the lung lesions were typical of pneumococcal pneumonia.

Dr Vandegrift I disagree with the diagnosis of pneumococcal pneumonia. The description and illustrations indicate a diagnosis of acute virus pneumonia. They are quite similar to the changes in a case of mine who died two days after an exacerbation with a spread to the left upper lobe. Microscopic examination showed edema and infiltration with neutrophils of the alveolar septae, fibrin lining of the alveoli, and a variable amount of purulent exudate in the alveoli which contained, in addition, edema fluid, macrophages and desquamated alveolar epithelium. In the entire right lung and left lower lobe were the usual findings of acute bronchitis, bronchiolitis and infiltration of the peribronchial tissue with plasma cells, lymphocytes and occasional eosinophiles.

Dr Van Metre Well, all that I can say is that perhaps you have one of the cases that we are looking for. Maybe the findings in your case and in the case under discussion are all that would be expected in an early case of influenza virus and pneumococcal pneumonia. However, on review of sections of my case with people more experienced than I—and sections were taken from all lobes—there was nothing

that could be seen in that pneumonia that could not be seen in pneumococcal infections. We believe from the clinical and virological standpoint that the virus may well have played an important role. I am delighted to hear that you have had a similar case.

Dr Arnold R Rich I think the studies that the speakers have carried on are immensely important because, from the pathological side, we have difficulty in knowing just what to think about the relation of influenza to pneumonia in non-pandemic times. Of course, during the pandemic influenza, the effects of the virus were visible and were seen by everybody. It was well recognized that the virus produced a mononuclear infiltration of the bronchial walls, and that the bacteria produced a purulent exudate in the bronchial lumen and in the alveoli. The studies of Shope have, of course, shown that very beautifully in swine influenza, too, and we are all familiar with the effect of another virus, that of measles, which also renders the body susceptible to bacterial pneumonia. There, too, the walls of the bronchioles and bronchi are infiltrated with mononuclear cells, and we recognize that as characteristic of the virus, with the superimposed bacteria producing the purulent exudate. Now the curious thing is that in non-pandemic influenza periods, even though influenza may be prevalent in the population, we very rarely see this bronchial infiltration with mononuclear cells in adults who die of pneumonia. It is a pity. I think that the question arises right away as to whether the influenza viruses that are present in non-pandemic times are different from the pandemic virus in that they do not call forth the mononuclear infiltration of the bronchial walls, or whether the pneumonia that we see outside of pandemic influenza periods is pneumonia that is not associated with influenza.

In relation to Dr Langmuir's studies, since influenza flourishes in periods in which other respiratory and bacterial infections are common, I would like to ask whether it would be possible to set up curves, similar to the one showing a parallelism between influenza and pneumonia, for other infections and pneumonia. Could the influenza-pneumonia correlation have been coincidence?

I would like to ask Dr Ward how often the influenza virus would be found, during an influenza epidemic, in the sputum of individuals who didn't have influenza. The possibility of terminal aspiration of sputum must be borne in mind in relation to the isolation of influenza virus from the lungs at autopsy. It is very common that a person who is dying aspirates his sputum. When influenza is wide spread in the population would it not be possible to find, in patients without influenza, influenza virus in the lung that had been aspirated terminally? I think that is an important point.

Some of you may remember that last year Dr Ward, Dr Van Metre, and the pathology department, at a Monday Clinical Pathological Conference, went over some of these cases, and we were not able to reconcile, as well as we would like to have done, the pathological picture with the findings of the virus studies. I repeat that the thing that bothers us is that in patients who died of pneumonia following the pandemic influenza mononuclear infiltration of the bronchial walls was so

widespread and so obvious, and it is so obvious in relation to measles, which also predisposes to pneumonia, but in epidemic influenza periods, adults dying from pneumonia do not show it, and we would like to know why. You have seen that in the adult case presented by Dr Van Metre influenza virus was isolated in high titer, but the bronchioles did not show the mononuclear infiltration. I think, from a study of the lesions in that case, that there is nothing there that one wouldn't see in an early pneumococcal pneumonia uncomplicated by influenza. Continued studies such as these will, I think, lead us to understand what these discrepancies really mean.

Dr Thomas B Turner I would like to make one or two comments which are somewhat tangential to the main question under consideration. First, I think it is obvious that, using these biological tests in the isolation of influenza virus, it is not possible to isolate virus in 100 per cent of the cases in which it is present. Second, two things of considerable interest were referred to in passing. Some of these isolations were made at inter-epidemic periods. We have all wondered what happens to influenza virus between epidemics, it is perfectly clear that the virus is present in the community in occasional cases in these inter-epidemic periods and that is perhaps the way it is kept alive. Further, a very important aspect is the great variation in antigenicity of these different strains. It is quite possible that the strains now included in influenza are not by any means the best immunizing strains to use. Perhaps after testing large numbers of strains one or two can be found which will be much more protective than the vaccine now available.

Dr Alexander D Langmuir Dr Rich has really put his finger on the toughest problem. Certainly one simple explanation for the increase in pneumonia with each influenza epidemic might be coincidence if we didn't have a considerable amount of other evidence to support a definite relation. I think of meningococcal infections as another example of bacterial infection spread by the respiratory tract. It has its own characteristic pattern. It recurs in seasonal waves over a period of five or six years, in a wholly different manner from pneumococcal pneumonia, and with no relation to influenza epidemics. I think of streptococcal infections, what little information we have of their incidence is measured by scarlet fever. Scarlet fever is a disease which occurs more frequently in the late winter and spring. In certain areas—apparently unpredictably, maybe every five, ten or twenty years—there is a rise with a rate four, five, eight, or ten times as high as in the preceding years, lasting for two or three years. Then it declines. This occurred in Baltimore and Washington during the war period, and the rates are now returning to a low level. The epidemiological patterns of bacterial respiratory diseases are each distinct and different. Only the pattern of pneumococcal pneumonia seems to bear a partial relation to epidemic influenza. I do not believe coincidence is an adequate explanation of the epidemiological observations.

Dr Rich What is the frequency with which influenza antibody is found in the population in a non-influenza period?

Dr Langmuir It is a span. It is a broad distribution from none to a very high

titer This was shown at Fort Bragg before the 1943 epidemic of type A Samples were taken from normal soldiers from every area of the country There had been no epidemic since 1940-41 According to our laboratory technique, about 38 per cent of the bloods titered 1 to 16 or less, about 40 per cent titered 1 32, and 20 per cent 1 64 or higher, something in that range After the epidemic we again, through the spring of 1944, collected bloods for a variety of reasons, and some of those were tested A much higher level of antibodies was found Forty to 50 per cent of specimens had titers 1 to 64 or more Apparently the antibody level in the whole country was raised There is no exact figure as to the number of individuals with antibodies in the general population It is rather a graded scale which varies in relation to epidemics

Dr Ward Dr Rich's question regarding the sputum from cases other than pneumonia reminds me of Mrs Maxwell's thesis which was written on this subject One section of that thesis is called "Defects of the Study" She points out effectively, it seems to me, that what we really need in this particular study are two or three types of answer—the first, how much influenza virus is there among individuals who are well during an influenza epidemic? That question is unknown and cannot be answered, the second, how many cases of diseases other than pneumonia may show virus, is equally unknown The autopsy material does indicate that there isn't as much as one might suppose Twenty-six of the lungs examined were from cases occurring during the influenza epidemic and none showed influenza virus We were able to test the nasal washings of 18 contacts of influenza and secured blood specimens from these individuals These contacts were interns, assistant residents and nurses on the wards where influenza cases were hospitalized From two of those individuals we secured virus A positive serologic response occurred in one of those two One of the questions Dr Fred Bang mentioned a few months ago in a similar seminar across the street bears along with Dr Rich's and Dr Langmuir's question How do you know if you tested you could not find other viruses, such as herpes? I don't know—possibly you might To follow that along another step, if we had some technique for measuring viruses which we think now produce mild respiratory diseases could we show that pneumococcal pneumonia was associated with these particular viruses in a certain proportion of cases?

With regard to Dr Rich's comment about mitral stenosis playing a part in the death of the second case I think it is of interest that Dr Taylor, in 1940, showed that if mice were infected with a sublethal dose of influenza virus and the virus was allowed to grow in the lungs for a couple of days, and then the lung was embarrassed either by inoculation of sterile fluid or simply reanesthetizing the mice, death was produced in the mice He went on to postulate that what may be occurring, in complex or mixed infections of influenza virus and pneumococci or other bacterial agent in man may be that the virus growing inside the cell produces no particular damage at that particular moment, but when the bacteria produce the edema fluid the virus is spread from one cell to another because the cell itself is embarrassed Here is a patient with mitral stenosis There is more fluid present

than in a patient who doesn't have mitral stenosis. We have a virus in the lung. That virus, therefore, following Dr Taylor's thesis along, may be able to spread simply because of the edema fluid in the lung as the cells are "embarrassed" by that edema fluid.

In response to the aspiration question, I think it entirely possible that influenza virus may be aspirated into the lungs just before death. In one of the cases presented tonight, E T, the lungs titer 10^{-8} in chick embryos. I believe such a titer too high to be explained on the basis of aspiration alone.

BOOK REVIEWS

(These reviews represent the individual opinions of the reviewers and not necessarily those of the members of the Editorial Board of this Journal)

Internal Medicine in General Practice, 2nd edition By ROBERT P McCOMBS
Illus 741 pp \$8 00 W B Saunders Company, Philadelphia, Pennsylvania,
1947

The doctor with a large general practice is the busiest man in medicine. He must, of necessity, see a great number of patients during the course of each day and seldom has time to sit and ponder over his problems. Frequently these are not great, but in many instances he sees the beginnings of serious disease and more likely than not he must follow these through. He must then be able to recognize them quickly and be competent to treat them. On the other hand, he seldom has the time for extensive reading on new subjects or even for review of the fundamentals of diagnosis. For such a person this book cannot be too highly recommended. It is short, very much to the point, and clearly and interestingly written. Stress is laid entirely on diagnosis and treatment, and controversial points are bridged over or left to the specialist. Many of the rarer diseases are only mentioned in passing, but all the fundamental ones are stressed. Especially helpful are the frequent summaries of differential diagnosis. These are extremely well done and very helpful in the rapid diagnosis which must often be made. There are also excellent summaries of the various important laboratory tests and aids with emphasis on the worth and limitations of each.

Finally, there are the sections on therapy. In this second edition, these have been brought up to date with the addition of the antibiotics and other newer drugs. In addition to covering these, the author has done an excellent job of "debunking" many of the drugs and compounds all too often and too freely used because of the persuasive literature received in the mail. This has been especially well done in the realm of vitamins and hormones.

In conclusion, this is a book which should be in every general practitioner's library. By reading it he can, in a relatively short time, review the fundamentals of internal medicine and at the same time become familiar with the newer concepts of diagnosis and treatment.

W B B

Selected Writings of Benjamin Rush, The Edited by DAGOBERT D RUNES
433 pp \$5 00 *Philosophical Library, New York, New York, 1947*

A selection from the writings of so many-sided a man as Benjamin Rush is a promising undertaking. The editor has grouped about forty of Rush's lectures, pamphlets, essays, letters, etc., together under the main headings of "On Good

Government," "On Education," "On Natural and Medical Sciences," "On Miscellaneous Things" The section on natural and medical sciences is the longest one and includes, among other things, sections on psychiatry, a field in which Rush's fame has been most enduring But even those chiefly interested in the physician Rush will welcome this opportunity of making the acquaintance of Rush the theologian, statesman, and educator For Rush's versatility has made him not only an outstanding figure in the political and civil life of the young and struggling United States, but also one of the most fascinating examples of the "enlightened" doctor

To the student of Rush, the present selection has the short-coming of giving the date but not the source of the pieces published An appended list of writings of Rush published during his lifetime is limited to mere titles and can, therefore, hardly be considered a substitute An additional bibliographical list, however, lists a number of publications which are helpful for further examination

O T

Practical Physiological Chemistry, 12th edition By PHILIP B HAWK, BERNARD L OSER, AND WILLIAM H SUMMERSON Illus 1323 pp \$10.00 *The Blakiston Company, Philadelphia, Pennsylvania, 1947*

This new edition of the former "Hawk and Bergem" has the names of Oser and Summermon on the title page Drs Hawk and Oser are both associated with Food Research Laboratories at Long Island City Professor Summermon is a member of the bio-chemistry department of Cornell University Medical College The preface by Hawk states that this edition "represents a complete revision of the subject matter of this book" He states that "new sections have been introduced on the polarograph, on isotopes, on the sulfa drugs, on metabolic antagonists and antibiotics, on the Warburg tissue slice procedure, on the theory and practice of photometric analysis, on the electrophoretic fractionation of the plasma proteins, on the composition of foods, and on the various vitamins Also many new quantitative procedures for blood and urine analysis have been added"

This volume like its predecessors, is an excellent book to be kept at the elbow of anyone performing chemical work in a biological field because it provides quick reference to a mass of chemical detail on chemical-biological subjects in a readable form It provides specific procedures and presents a certain amount of chemical and biological background for them Considering the complex data in this field, the book provides only rather arbitrary tests and reasons for same References and background evidence are not emphasized This means that the significance of the chemical steps or of the biological application must be sought largely elsewhere It is difficult for the reviewer to make a statement about accuracy except to state that in previous volumes he has found relatively few distinct errors

F W B, Jr

BOOKS RECEIVED FOR REVIEW

- Acute Bacterial Diseases, The* By HARRY F DOWLING Illus 463 pp \$6 50
W B Saunders Company, Philadelphia, Pennsylvania, 1948
- Care and Managment of Laboratory Animals, The* Edited by ALASTAIR N WORTON Illus 268 pp \$8 50 *The Williams & Wilkins Company, Baltimore, 1947*
- Clinical Diagnosis by Laboratory Methods* By JAMES C TODD, ARTHUR H SANFORD, AND GEORGE G STILWELL Illus 954 pp \$7 50 *W B Saunders Company, Philadelphia, Pennsylvania, 1948*
- Dissection of the Cat, The* By BRUCE M HARRISON Illus 109 pp \$3 50
C V Mosby Company, St Louis, Missouri, 1948
- Handbook of Treatment and Medical Formulary* By CHARLES M GRUBER 594 pp \$7 00 *F A Davis Company, Philadelphia 3, Pennsylvania, 1948*
- Human Physiology*, 3rd edition By F R WINTON AND L E BAYLISS Illus 592 pp \$7 00 *The Blakiston Company, Philadelphia 5, Pennsylvania, 1948*
- Identification of Tumors* By N CHANDLER FOOT Illus 397 pp \$6 00 *J B Lippincott Company, Philadelphia, Pennsylvania, 1948*
- Noah Webster Letters on Yellow Fever Addressed to Dr William Currie* 110 pp \$2 00 *The Johns Hopkins Press, Baltimore, Maryland, 1947*
- Treatment by Diet*, 5th edition By CLIFFORD J BARBORKA Illus 784 pp \$10 00 *J B Lippincott Company, Philadelphia, Pennsylvania, 1948*

THE NITROGEN METABOLISM OF GRAM-POSITIVE BACTERIA*

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- I THE ASSIMILATION OF AMINO-ACIDS
- II INTRACELLULAR UTILIZATION OF GLUTAMIC ACID AND ITS INHIBITION BY CERTAIN ANTIBACTERIAL AGENTS
- III THE NATURE OF PENICILLIN SENSITIVITY IN STAPHYLOCOCCUS AUREUS

LECTURE 1

THE ASSIMILATION OF AMINO-ACIDS

The growth of any cell involves an increase in its parts. One of the most important constituents of the bacterial cell is protein and the growth of a bacterial cell involves the synthesis of a wide variety of structural and functional proteins within the cell. Analysis of the amino-acid composition of bacterial proteins (1, 2) shows that these proteins resemble those of all other cells in being built up from some twenty-odd different amino-acids. Consequently the synthesis of protein by the growing cell requires the provision of all the necessary amino-acids in the correct proportions. Such provision can be made in one of two ways: by synthesis, or by assimilation of the ready-formed substances from the environment. The synthetic abilities of bacteria can be tested by investigation of their nutritional requirements, and such investigations reveal wide differences between various species and strains. The autotrophic organisms can synthesise all their amino-acids from very simple inorganic substrates such as CO_2 and ammonia, some heterotrophic bacteria can synthesise all their requirements from ammonia as N-source and a single C-source such as glucose but other bacteria of the heterotrophic group cannot grow unless they are supplied with certain amino-acids in the ready-formed state. The synthetic disability may be simple, such as that of *Eberthella typhosa*.

* Three Hertel Lectures delivered at the Johns Hopkins Hospital, March 22-24, 1928

which requires tryptophan, or complex such as that of *L. casei* or *Strep. haemolyticus* strains of which may require as many as 20 amino-acids. In these cases synthesis of certain amino-acids is impossible and growth—i.e. protein synthesis—can occur only if the organisms can assimilate the essential amino-acids from their external environment.

A number of workers have turned their attention to the problem of how these nutritionally-exacting organisms have arisen. Their present nutritional requirements restrict their habitat to environments rich in amino-acids and, in many cases, growth factors, environments which are to be found naturally only in biological fluids. Knight (3) and Lwoff (4) are among those who have suggested that the exacting organisms have developed from non-exacting ones, that if a non-exacting organism assumes a parasitic existence or becomes established in a biological fluid then growth may occur more rapidly by the utilisation of essential substances already present in the environment than by synthesis of these substances, and that the ability to synthesise such substances will then be gradually lost through disuse. Weight is lent to this hypothesis by the demonstration that non-exacting organisms can be derived from exacting ones if the latter are treated to a process of attrition. Thus if *Eberthella typhosa* is subcultivated serially into media containing progressively less and less tryptophan it will eventually be able to dispense with added tryptophan and synthesise its complete requirement (5), while strains of *Staph. aureus* initially needing ten or more amino-acids can, by a similar process, be trained to synthesise all their amino-acid requirements from ammonia (6).

If the ability to synthesise an amino-acid is lost, then the organism must be able to assimilate that amino-acid in order to grow. It is with this process of assimilation that I wish to deal mainly today. In essence the process involves the removal of an amino-acid from the external environment and its passage into the cell into the region where its further metabolism may be accomplished.

The work started, as so much scientific work does, from an investigation into a little-related problem. In the course of our studies at Cambridge into the amino-acid metabolism of bacteria, we found that some bacteria grown under certain conditions are able to produce enzymes which quantitatively and specifically decarboxylate certain amino-acids to their corresponding amines (7). These enzymes, which

can be prepared in a specific state and whose action can be studied easily by the manometric method, provide an accurate and rapid micro-method for the estimation of their substrates (8, 9) In each case the enzyme will attack the specific amino-acid substrate only provided that three polar groups are free the —COOH group, the $\alpha\text{—NH}_2$ group, and a third polar group remote from the —COOH group attacked, in consequence the enzymes estimate the free amino-acid substrate only We were using these enzymes to investigate the amino-acid composition of a variety of bacteria by analysis of acid-hydrolysates of washed suspensions of the organisms concerned It occurred to us that a certain amount of free amino-acid might in some cases be carried down from the growth medium either on the outside or within the cell suspensions and that this would invalidate values obtained after acid hydrolysis To check whether this was the case, we divided our cell-suspensions into two parts, one of which was subjected to acid hydrolysis as usual and the other was disintegrated by mechanical agitation with glass particles The two preparations were then analysed by the decarboxylase method Results fell into two groups, in the first group, which comprised organisms such as *Escherichia coli* and *Aerobacter aerogenes*, the amount of free amino-acid found in the disintegrate was negligible when compared with that obtained after hydrolysis, in the other group, which comprised organisms such as *Staph aureus* and *Strep faecalis*, an amount of free amino-acid was found in the disintegrated cell which amounted in some cases to as much as 50% of the total amino-acid liberated after hydrolysis

The position is made clear by fig 1 In this case we take a thick suspension of well-washed cells of *Strep faecalis* grown in a casein-digest medium and use it as substrate for glutamic acid decarboxylase, it can be seen that the addition of the enzyme is followed by a small evolution of gas (carbon dioxide) which corresponds to free glutamic acid carried down with and on the outside of the cells during harvesting and washing If, however, an equivalent sample of cells is mechanically disrupted, then the glutamic acid assay is greatly increased This extra glutamic acid must be liberated by the disruption of the cell-wall Similar results are obtained if the cell-wall is broken by heat denaturation or by treatment with detergent substances (10, 11)

At this point it may be useful to define the terms in which concentra-

tions of amino-acids, etc will be stated and discussed in these lectures. All estimations of amino-acids have been made by the manometric method in which the evolution of CO_2 equivalent to the amino-acid present is measured, consequently it is convenient to discuss the results in terms of the gas evolution in each case i.e. in μl . This means that 1 m mol of amino-acid is equivalent to $22.4 \times 10^3 \mu\text{l}$ or, alternatively, concentrations can be converted to molecular quantities such as μmols by division of the values by 22.4

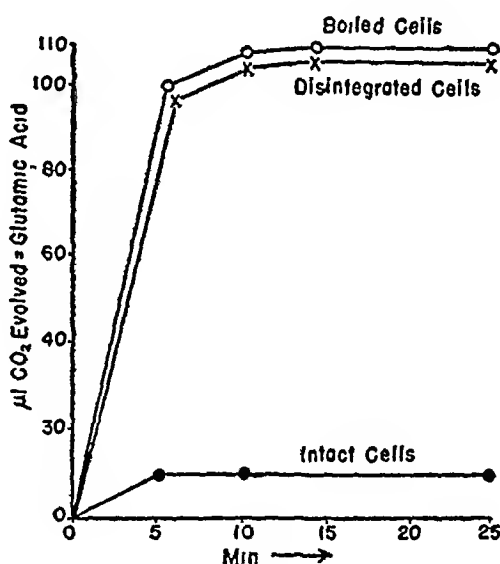


FIG 1 LECTURE I Action of glutamic acid decarboxylase on intact and disrupted cells (*Strep faecalis*) Each estimation carried out on 60 mg dry weight of cells.

The experiment just described demonstrates that cells of *Strep faecalis* contain free glutamic acid in their internal environment, 100 mg dry weight of cells contain approx 170 μl free glutamic acid. Similar investigations with other enzymes of the decarboxylase series show that the cells also contain lysine, histidine and ornithine in the free state within the internal environment but, in *Strep faecalis*, no free arginine or tyrosine. The absence of these last two amino-acids is probably due to the fact that the cells possess enzymes which bring about their rapid decomposition—arginine dihydrolase and tyrosine decarboxylase. Other amino-acids almost certainly exist in the free

state within the cell but absence of suitable techniques for their estimation has made it impossible to investigate this possibility up to the present. A question that immediately arises is—can free amino-acids be demonstrated within the internal environment of all cells? You will remember that, in the early investigations on amino-acid composition, some cells contained free amino-acids and others did not. Consequently we started an investigation of a variety of organisms, they were all grown in casein-digest (as a growth medium rich in free amino-acids) with glucose and such specific growth substances as might be required by exacting organisms, they were then well-washed and their

TABLE 1 (LECTURE I)

*Internal Concentration of Glutamic Acid*Values expressed in μ l glutamic acid (internal)/100 mg cells

<i>Staph aureus</i> 563	820	<i>Esch coli</i> 86	0
<i>Staph aureus</i> 6773	635	<i>Esch coli</i> EST	0
<i>Staph aureus</i> D	456	<i>Esch coli</i> 7020	0
<i>Strep faecalis</i> ST	230	<i>Aerobact aerogenes</i> I	0
<i>Sarcina lutea</i>	225	<i>Aerobact aerogenes</i> II	0
<i>M lysodeikticus</i>	203	<i>N catarrhalis</i>	0
<i>L casei</i> YCT	174	<i>B brevis</i>	0
<i>Strep faecalis</i> SF	107	<i>Proteus vulgaris</i>	0
<i>Strep haemolyticus</i> R	99	<i>Ps pyocyanea</i>	0
<i>Cl sporogenes</i> Bellette	81	<i>Bact paracoli</i> 6578	0
<i>L delbrueckii</i> B	80	" <i>Staph PT</i> "	0
<i>L helveticus</i> B	74		
<i>B subtilis</i>	20		
<i>B mesentericus</i>	14		

lysine and glutamic acid content investigated before and after rupture of the cell-wall. Table I shows results obtained for the free glutamic acid content (12). The organisms fall into two well-defined groups, those which possess free amino-acids in the internal environment, and those which do not. A further correlation is immediately obvious, all those cells in the first group are Gram-positive while those in the second group are Gram-negative and no exception to this rule has yet been discovered. The ability of the Gram-positive cells to accumulate free glutamic acid varies widely, within our experience the species which can, in general, effect the highest internal concentration of glutamic acid is *Staph aureus*. At the other end of the scale we get organisms

such as *B subtilis* and some of the *Clostridia* which contain small but still definite amounts of free glutamic acid within the cells. The yeasts, also Gram-positive, are interesting in that they accomplish a high concentration in the internal environment of all six of the amino-acids that we can study by this method. But however it is grown or tested, and we have tried many things, it has not yet been possible to demonstrate any free amino-acid inside the Gram-negative cell.

Most of the work I shall be describing to you has been carried out either with *Strep faecalis* or with *Staph aureus*, both of which can effect a high concentration of both glutamic acid and lysine but, as we shall see later, apparently do so by different mechanisms. The existence of the free amino-acid within the cell in these cases enables us to investigate the metabolism of that free amino-acid by a study of the conditions which affect the level of the free amino-acid within the cell.

In the first place, the level within the cell depends to a certain extent on the level in the external environment. The estimations described so far were carried out on cells which had been grown in an external medium rich in amino-acids. The quantity of amino-acid in the medium can be cut down considerably without preventing growth and a reasonable amount of organism can be obtained in a medium containing 0.1% Marmite as sole source of amino-acids. Such a medium we call our "deficient" medium although it is not deficient in that any particular growth essential is missing—they are merely there in minimal quantities rather than in gross excess as in the casein-digest medium. *Strep faecalis* cells grown in this deficient medium have much less free glutamic acid or lysine within the cells than when they are grown in the rich medium. This then enables us to establish a useful technique by growing our organisms in a deficient medium we can produce cells which possess little free amino-acid in the internal environment, by taking such cells and placing them in an external environment rich in free amino-acid we can investigate the conditions whereby such amino-acids pass across the cell-wall and accumulate within the cell. Alternatively we can grow cells in an amino-acid-rich medium, place them in an amino-acid-free environment and study the conditions under which the internal amino-acids will pass out of the cell.

Let us take the second case first, since it might affect the conditions of our estimations. If we take cells containing a high internal concen-

tration of either lysine or glutamic acid and stand them in either distilled water or a suitable salt solution at 0° or 37° then neither lysine nor glutamic acid can diffuse out of the cell unless autolysis takes place. This holds true over the pH range 5.5–8.5 although some leakage occurs outside that range—possibly due to damage to the cell-wall under these conditions. Our amino-acid estimations by the manometric method take about 15 min so that the technique described for the estimation of internal and external amino-acids is not invalidated in any way by leakage across the cell-wall during estimation.

Now let us turn to the opposite aspect—the migration of amino-acids from external to internal environment. I intend to deal with two amino-acids only in detail—lysine and glutamic acid. Substances can pass across a cell-wall by two processes, either by diffusion or as a result of an active process by the cell. The evidence suggests that lysine passes across the cell-wall by diffusion while glutamic acid enters the cells as part of an active process (11).

If cells deficient in lysine are placed in a solution of lysine in a salt medium, then lysine begins to enter the cell immediately the latter comes into contact with its new environment and enters the cell very rapidly until the new level inside is attained. In the case shown the experiment was carried out at 10° and the new level within the cell is attained within 10 min of contact. The migration occurs equally well at 0° and is unaffected by any of the common inhibitors of metabolism such as HCN, iodoacetate, etc.

If deficient cells are placed in a solution of glutamic acid or glutamine, no entry into the cell takes place even at 37° over a period of 3 hr. If glucose is added to the external medium then fermentation takes place and glutamic acid (or glutamine) enters the cell until a new level in equilibrium with the external environment is reached. *Strep faecalis* cells have restricted catabolic activities but can also attack arginine by the enzyme arginine dihydrolase (13) and it appears that arginine can also act as a source of energy for the migration of glutamic acid although its efficiency as energy source is much less than that of glucose. The migration in the presence of glucose is inhibited by iodoacetate or sodium fluoride in concentrations inhibitory to glycolysis. Glucose can, under certain conditions, be substituted by adenosine-tri-phosphate as energy source.

The difference between the two processes can be further shown if we study the rates at which the two amino-acids enter deficient cells. The rate of entry of lysine is roughly proportional to the external concentration over the range studied, whereas the rate of entry of glutamic acid reaches a maximum when the external concentration approaches 50 $\mu\text{l/ml}$. The curve obtained for glutamic acid is similar to that obtained for the variation of the rate of an enzyme reaction with substrate concentration while that obtained for lysine is consistent with a diffusion process in which the driving force consists of the difference in concentration between internal and external environments.

The effect of temperature also differentiates the two processes. The rate of entry of lysine increases linearly with temperature and the temperature coefficient over the range 20–30° is 1.40 which is within experimental error of the value obtained for the free diffusion of lysine. The rate of entry of glutamic acid varies in a completely different manner with temperature and the temperature coefficient of approx. 2 over the range 20–30° is again typical of an enzymatic reaction.

It would be of considerable interest to know which amino-acids other than lysine and glutamic acid enter the cell by diffusion or by an active process but we are at present handicapped in such a search by the absence of really suitable methods of investigation for most other amino-acids. We can differentiate between those amino-acids which can pass into washed deficient cells at 37° in the absence of a source of energy and those which can only do so in the presence of a source of energy such as glucose. Histidine passes into the cell to a limited extent in the absence of glucose and to a markedly greater extent in the presence of glucose. Aspartic acid, as judged by the rather non-specific chloramine-T estimation, only enters the cell as a result of an active process. Arginine cannot be shown to enter the *Strep faecalis* cell at all by this method—probably because arginine dihydrolase removes any accumulated excess. Of the amino-acids that we can estimate, only lysine appears to enter the cell by simple diffusion. The same general findings hold good for *Staph aureus*, lysine enters by diffusion, glutamic acid as the result of an active process. In yeasts we find that all the amino-acids tested, including lysine, can enter the cell only while glucose is being metabolised and all the amino-acids, including arginine and tyrosine, accumulate at high concentration within the internal environment (14).

Returning again to *Strep faecalis* we find that both lysine and glutamic acid enter the cell, under suitable conditions, until the internal concentration is markedly higher than that holding in the external environment. By direct measurement of the volume of the cell and calculation of the free space within the cell, we can get some idea of the ratio between internal and external concentrations. In the case of lysine the internal concentration in *Strep faecalis* varies from 2-3 to 15-20 times the external concentration over the range studied (25-400 $\mu\text{l/ml}$), the lower the external value, the higher the relative concentration within the cell. In the case of glutamic acid, the ratios reach 50-60 in low external concentrations while the actual concentration within the cell may reach 0.06M glutamic acid. These values may be considerably exceeded in the case of some strains of *Staph aureus* which have greater capacity for the internal concentration of glutamic acid and where an internal concentration of 0.18M free glutamic acid has been measured and a ratio of internal to external concentration of 400 obtained.

The entry of both lysine and glutamic acid into the cell is made against the concentration gradient. Biophysicists tell us that there are two conditions under which such a migration can occur

- 1 That the cell-wall is permeable to the substance concerned and that this is held by electrical or chemical combination on the side of greater concentration

- 2 That the cell-membrane is impermeable to the substance concerned and that migration across the membrane takes place in a different physical or chemical state from that which accumulates on one side

The evidence we have considered so far indicates that lysine diffuses freely into deficient cells but does not pass out of these cells if they are placed in a lysine-free medium. Under physiological conditions lysine is positively charged while the bacterial cell is negatively charged, consequently there is an electrical attraction between the cation and the cell. It seems probable that lysine enters the cell by diffusion and is held therein by electrical combination. We have attempted to get some light on this point by investigations of the electrophoretic mobility of the cells in the presence and absence of lysine (15) and it has been possible to show that there is a definite decrease in the negative surface charge of deficient cells when brought into contact with lysine.

However there is other evidence that the situation is not as simple

as this You will remember that lysine enters the deficient *Strep faecalis* cell at 37° in the absence of glucose, if glucose is added to the system during assimilation then the entry of lysine is reduced and the level attained within the cell may be reduced to 10% of that attained in the absence of glucose Likewise if cells saturated with lysine are incubated in water, no outward diffusion of lysine takes place, if however glucose is added to the external medium a rapid outward diffusion takes place until the internal level is adjusted to a lower level If the lysine is held within the cell by a negative charge, it would appear that the presence of glucose has had the effect of discharging the cell, electrokinetic measurements however show that the negative charge on the surface of the organism is not affected by the presence of glucose We do not know, of course, what is happening within the cell but it would seem that, if the lysine is in fact held by an internal negative charge, then that charge must arise from some specific centre—such as an ion which migrates out of the cell during glycolysis—rather than the simple net charge on the cell as a whole

If we study the effect of the pH of the external medium on the rate of entry of lysine into the cell, we get a curve showing that the rate increases with rising pH to the limit of alkali tolerance at pH 9.5 Since the isoelectric point for lysine is 9.47 it appears that the amino-acid crosses the cell-wall most readily in the isoelectric state The final concentration attained within the cell is not markedly affected by the external pH, the level attained at an external pH 8.5 being 14% higher than that attained at an external pH 5.0 It seems probable that lysine passes across the cell-wall in an isoelectric state but accumulates within the cell in the cationic state It is obvious that we need much more knowledge concerning the ionic balance within the cell and the ionic exchanges that occur during metabolism before we can obtain any true understanding of lysine assimilation

If we turn to the assimilation and internal accumulation of glutamic acid, we have to admit that the position is even less satisfactory This amino-acid enters the cell not by diffusion but as a result of some energy-requiring process The amino-acid exists in an anionic state in physiological conditions and is therefore entering the cell against the electrostatic gradient and against the concentration gradient Electrophoretic measurements show that the surface charge on deficient *Strep faecalis* cells is unaffected by the presence of glutamic acid either

alone or in the presence of glucose although assimilation will take place in the latter case. These negative results lend weight to the electro-diffusion hypothesis for lysine assimilation where the addition of lysine is followed by a definite reduction in the charge on the organism. With *Strep faecalis* cells, the internal and external glutamate concentrations are in true equilibrium as long as a source of energy such as glucose is present: if deficient cells are incubated in high concentrations of glutamic acid, then the amino-acid enters the cell until equilibrium is established, if saturated cells are incubated in glutamic-acid-free media, then glutamic acid leaves the cell until equilibrium is established and, for a given external concentration, the same internal concentration is reached whether the amino-acid is passing into or out of the cell. No diffusion in either direction takes place in the absence of exergonic metabolism. It would seem that we may have here a case where the cell-wall is impermeable to the substance concentrated within the cell—and, it may be, that the substance crossing the cell-wall is not glutamic acid itself.

The first possible migrant substance we tried was glutamine. There is a certain amount of evidence that this may be involved. If the rates at which glutamine and glutamic acid pass into the deficient cell are studied, then glutamine is often found to pass more rapidly while glutamic acid may show a short lag phase after introduction of the cells before it begins to enter the cells. But glutamine again does not give rise to an internal concentration in the absence of glucose, in the presence of glucose, glutamine and glutamic acid give rise to the same internal concentration. Estimations of the nature of the internal substance under such conditions indicate that it is a mixture of glutamic acid and glutamine containing 20–40% glutamine. It has been shown recently in this country by Waelsh *et al* (16) that methionine sulphoxide competitively blocks the utilisation of glutamic acid by certain *Lactobacilli* and is non-competitively neutralised by glutamine. This suggests that methionine sulphoxide inhibits the formation of glutamine from glutamic acid. Elliott (17) has now shown that the first stage in the synthesis of glutamine involves a phosphorylation of glutamic acid, in the presence of adenosine-tri-phosphate, and we (18) have shown that the enzyme concerned is competitively inhibited by methionine-sulphoxide.

The assimilation of glutamic acid by *Strep faecalis* requires glucose

but will also take place if large amounts of adenosine-tri-phosphate are added (approx 30-50 molecules of ATP are required to promote the assimilation of 1 molecule of glutamic acid) Further, the assimilation in the presence of either glucose or ATP is competitively inhibited by methionine sulphoxide while that of glutamine is unaffected This clearly suggests some role of glutamine or a like substance in glutamic acid assimilation However glutamine itself still requires either glucose or ATP to migrate across the cell-wall and would appear to be ruled out as the migrant substance But a phosphorylated derivation of glutamic acid may be an intermediate step in the synthesis of substances other than glutamine - glutathione and vitamin B₆ conjugate appear probable for example Glutathione has been tested as the migrant substance but does not give rise to either glutamic acid or glutamine inside the cell and our knowledge of the impermeability of cell-membrane towards acyl-phosphates would suggest that "glutamyl-phosphate" itself could not penetrate into the cell There is, however, definite evidence pointing to the idea that a metabolite of glutamic acid is the form in which this amino-acid crosses the cell-wall

However, when we try to extend these results to other organisms we find that we do not always get the same picture The process of glutamic acid assimilation in *Staph aureus* possesses several features which suggest that it is different from that in *Strep faecalis* Glutamic acid does not pass into deficient *Staph aureus* cells in the absence of a source of energy such as glucose In the case of *Strep faecalis* no leakage of internal glutamic acid takes place in the absence of glucose while, in the presence of glucose, there is a migration across the cell-wall in either direction until equilibrium is established In the case of *Staph aureus* however there is a slow leakage of glutamic acid out of the cell in the absence of glucose and the addition of glucose checks rather than enhances this leakage—the picture being that the glycolysis is holding the amino-acid inside the cell in opposition to the outward diffusion process rather than establishing a new equilibrium across the cell-wall In the case of *Strep faecalis*, methionine sulphoxide competitively inhibits the assimilation process, in *Staph aureus* it has no effect on assimilation even when present in a concentration 200 times that of the glutamic acid in the external medium

A possibility which we have not considered yet is that of ionic

exchange A secretion process may sometimes consist of the active transfer of one ion across a membrane to substitute for another ion of like charge which passes across the membrane in the opposite direction It may be that glutamic acid passes into the cell replacing some other negative ion which passes out of the cell Hotchkiss (19) has shown that *Staph aureus* cells accumulate phosphate within the cell in high concentration and that the amount of phosphate within the cell is reduced if amino-acid assimilation takes place He points out that during glycolysis an uptake of both nitrogenous and phosphate compounds takes place but that as more amino-acids are added to the external medium—as the medium becomes more complete in the nutritional sense—then more nitrogenous material is taken up and less phosphate This might mean that glutamic acid, and possibly other amino-acids, enter the cell in replacement of phosphate ions However, phosphate is involved in so many reactions within the cell and, as McIlwain (20) has shown, glycolysis itself is increased after assimilation of glutamine so it is difficult to interpret these results with any certainty at present

The last question with which I wish to deal today is the nature of the boundary in the cell which holds the internal concentration of amino-acid and maintains the difference in concentration between internal and external environments The work of Hotchkiss (21) on the action of the antibiotic Tyrocidin and of other detergent substances enabled us to obtain some information on this point Hotchkiss observed that if cells of *Strep faecalis* or *Staph aureus* were treated with such detergents, then nitrogenous and phosphorus-compounds were liberated into the external medium It seemed highly probable that the nitrogenous material liberated would include these amino-acids which were held within the cell Fortunately it proves very easy to demonstrate this fact since the amino-acid decarboxylase preparations are not inhibited by high concentrations of tyrocidin and other detergents

This makes it possible for us to use the manometric method to study the action of tyrocidin a thick washed suspension of cells is placed in the main cup of the manometer and glutamic decarboxylase added from one side-bulb at the beginning of the experiment When the external glutamic acid has been assayed and removed, tyrocidin

is added from a second side-bulb and the release of glutamic acid from the cell can be seen to start immediately. The total internal free amino-acid can be judged from the assay with boiled cells and it can be seen that complete lysis occurs within 5-10 mins of the addition of the tyrocidin (11)

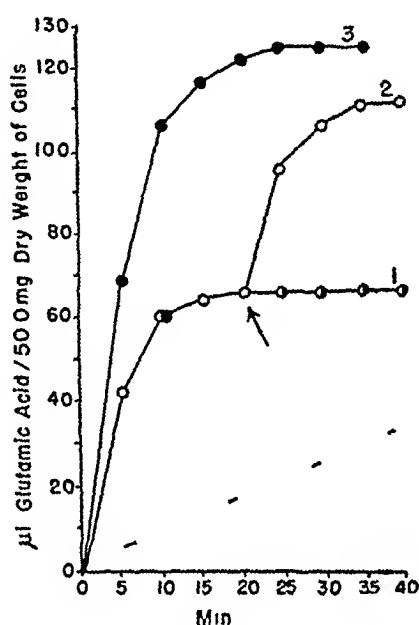


FIG 2 LECTURE I Relation between quantity of tyrocidin added and amount of glutamic acid released from internal environment. Dry weight of *Strep faecalis* cells assayed = 50.0 mg. Temperature = 30°. Manometer vessels made up with 1.0 ml M/5-phosphate pH 6.0, 1.0 ml washed suspension of *Strep faecalis* cells, and 0.5 ml glutamic decarboxylase (main compartment) with tyrocidin in side-bulbs. Manometers allowed to equilibrate and liberation of external glutamic CO₂ to cease before time = 0. Curve 1 0.5 mg tyrocidin tipped at time = 0. Curve 2 0.5 mg tyrocidin tipped at time = 0, further 0.5 mg tyrocidin tipped at time = 20 min (arrow). Curve 3 1.0 mg tyrocidin tipped at time = 0.

A very interesting thing is the effect of detergent concentration, if an amount of tyrocidin is added equal to half that required to liberate all the amino-acid, then we find that only half the total amino-acid is liberated—not, as one might expect, that all the amino-acid is liberated at half the speed. If we add a second quantity of tyrocidin after the effect of the first has finished, we find that the rest of the amino-acid is liberated. These results can be correlated with viable

counts if an amount of tyrocidin is added sufficient to release all the amino-acid, then all the cells are killed, if half this quantity is added, then half the cells are killed. From quantitative considerations it can be calculated that about 10^8 molecules of tyrocidin are required to kill one *Strep faecalis* cell.

The action of these detergents is apparently to modify the cell-wall in such a way that the concentration boundary is broken. The electron microscope has long revealed that most bacteria have a definite cell-wall although this is not easy to demonstrate in some of the Gram-positive cocci. However, electron-micrograph studies of *Strep faecalis*, *Staph aureus* and yeast cells before and after treatment with tyrocidin show clearly that the action of the detergent is to strip off the outer cell-wall (22). The cells are not completely lysed as they retain their shape and under the dark-ground appear little altered apart from a slight change in the clarity of the outline. This suggests either that there is some containing-membrane within the cell-wall which is not broken by the detergent action, or, alternatively, that the cytoplasm of the cell is in a gel form.

Phenol releases amino-acids from the cells in the same manner as tyrocidin although the action in this case is much slower and electron-micrographs again demonstrate a stripping of the cell-wall after phenol-action. We have endeavoured to determine the structure of the material stripped from the cell by phenol—using phenol since it is easily and quantitatively removed from the preparation by drying *in vacuo*. The material obtained by phenol treatment of large amounts of *Strep faecalis* contains about 50% nitrogenous material consisting largely of free amino-acids released from inside the cell, some phosphorous-compounds also largely released from the cells and approximately 40% ether-soluble, saponifiable material which probably comes from the cell-wall itself.

To summarise our main conclusions from the work studied so far, we find that Gram-positive bacterial cells assimilate and accumulate certain amino-acids in the free state within the internal environment, that some amino-acids, notably lysine, pass into the cell by diffusion but others, notably glutamic acid, can only enter the cell as a result of an active process, that the equilibrium concentration attained within the cell is markedly greater than that holding in the external environ-

ment, and that this concentration gradient is maintained at the cell-surface by a cell-wall probably consisting largely of lipid substances which can be dissolved away from the cell by certain detergent substances

In the next lecture, we shall proceed to discuss what happens to the glutamic acid after assimilation and concentration within the Gram-positive cell

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LECTURE 2

THE INTRACELLULAR UTILIZATION OF GLUTAMIC ACID AND ITS INHIBITION BY CERTAIN ANTIBACTERIAL AGENTS

Glutamic acid appears to occupy a key position in microbial metabolism It forms the main substrate for the transaminase systems

demonstrated in bacteria (1, 2) and, as such, is involved in the synthesis of aspartic acid and alanine. Bonner (3) has demonstrated by means of the biochemical mutant technique that it is the biosynthetic precursor of proline, ornithine, citrulline and arginine in *Penicillium*. Synthesis of the *L. casei* factor has shown this to be pteroylglutamic acid (4) which is one of a group of substances, known as the folic acid complex, which differ from each other in the number of glutamyl-residues they contain, thus the *casei* "fermentation factor" is pteroyl-triglutamic acid and vitamin B₁₂ conjugate contains pteroylheptaglutamic acid. Ratner, Blanchard & Green (5) have isolated a peptide from yeast which contains p-aminobenzoic acid and 10 or 11 glutamyl-residues in addition to an unidentified amino-acid. Glutamic acid forms part of the structure of Glutathione, discovered by Hopkins (6) and which has been demonstrated to act as a growth essential for *N. gonorrhoeae* (7). It is probably one of the constituents of Strepogenin (8) and forms 8-9% of the dry weight of bacterial protein (9).

In the first lecture I showed that glutamic acid is assimilated by Gram-positive bacteria and is concentrated in the internal environment. There is no doubt but that the glutamic acid within the cell must enter into metabolism of the cell and since our method of estimation assays only the free unsubstituted amino-acid, it follows that the level of glutamic acid measured within the cell must represent a balance between the rate at which it is assimilated from the external environment and the rate at which it is metabolised in the internal environment. It further follows that if the cell is treated in such a way as to inhibit the internal metabolism, then the level of glutamic acid will rise within the cell until it reaches the value which represents true equilibrium with the external environment.

When a survey of the action on assimilation of a variety of antibacterial substances was undertaken, it was found that the presence of crystal violet, or other basic dyes of the triphenylmethane series, resulted in a marked rise in the internal level of glutamic acid during assimilation by washed *Strep. faecalis* cells under otherwise standardised conditions.

The development of the dyes of the triphenylmethane series arose from a suggestion made by Paul Ehrlich, the father of chemotherapy

and the first Herter lecturer. He was struck by the specific staining affinities for particular tissues displayed by certain dye-stuffs and suggested that it should be possible to obtain dyes which would specifically stain, and thereby inactivate, parasitic protoplasm without affecting the host protoplasm. The suggestion was followed up by several laboratories and led indirectly to the development of the sulphonamides, through Prontosil, and to Bayer 205 (suramine). It did not reach any resounding success in the field of dye-stuffs proper although dyes of the triphenylmethane series have had limited clinical application.

The fundamental structure of the series is the tri-phenylmethane nucleus with either di- or tri-amino-substitution, acidic derivatives having no antibacterial activity. The carbinol base has no biological activity but if it is heated with hydrochloric acid, a quinonoid derivative is produced which is coloured and has antibacterial action. If the dye is reduced to the leuco-base, which is colourless, the activity is lost. Anti-bacterial activity thus appears to be associated with the quinonoid form and may be associated, as Kumler (10) has suggested, with the resonance that occurs in this form. The simplest dye of the series is p-rosaniline or triaminotriphenylmethane, substitution of alkyl groups in the amino-groups leads to a series of dyes of varying biological activity. The first studies of the series were by Kligler (11) who showed that the antibacterial activity increases as the degree of alkyl substitution increases. Thus crystal violet with six methyl-groups is about 100 times as effective as p-rosaniline with no methyl-groups, but the further substitution of a seventh methyl-group to form a quaternary-N compound, methyl green, results in loss of all biological activity. The varying activities are shown in Table 1 for the case of *Strep faecalis* as test organism, the increasing activity with increasing alkyl substitution is shown for both tri- and di-amino-triphenylmethane series, the most active dye of those tested is Brilliant Green which contains four ethyl substituents.

Fig 1 shows the effect of crystal violet on the assimilation of glutamic acid by *Strep faecalis* cells. The cells were grown and tested as I described yesterday and the diagram shows the rate of appearance of glutamic acid within deficient cells when brought into contact with a high concentration of glutamic acid in the presence of glucose with

or without Crystal Violet In the presence of the dye we find that the rate of uptake is slightly slower than in the untreated cells but that the final level is markedly higher in the dyed cells than in the untreated

TABLE 1 (LECTURE II)

Inhibition of growth of Strep. faecalis by dyes of the triphenylmethane series

NAME	SUBSTITUENT GROUPS	CONCENTRATION OF DYE							
		1/10,000	1/50,000	1/100,000	1/200,000	1/300,000	1/500,000	1/700,000	1/1,000,000
p Rosaniline	$-\text{NH}_2, -\text{NH}_2$ $-\text{NH}_2$	-	-	+	+	+	+	+	+
Euchsin	$-\text{NH}_2, -\text{NH}_2$ $-\text{NH}_2, -\text{CH}_3$	-	-	+	+	+	+	+	+
Methyl Violet	$-\text{N}(\text{CH}_3)_2$ $-\text{N}(\text{CH}_3)_2$ $-\text{NH CH}_3$	-	-	-	-	-	+	+	+
Crystal Violet	$-\text{N}(\text{CH}_3)_2$ $-\text{N}(\text{CH}_3)_2$ $-\text{N}(\text{CH}_3)_2$	-	-	-	-	-	-	+	+
Malachite Green	$-\text{N}(\text{CH}_3)_2$ $-\text{N}(\text{CH}_3)_2$	-	-	-	-	-	-	-	+
Brilliant Green	$-\text{N}(\text{C}_2\text{H}_5)_2$ $-\text{N}(\text{C}_2\text{H}_5)_2$	-	-	-	-	-	-	-	+
Methyl Green	$-\text{N}(\text{CH}_3)_2$ $-\text{N}(\text{CH}_3)_2$ $-\text{N}(\text{CH}_3)_2\text{Cl}$	+	+	+	+	+	+	+	+
Soluble Blue	$3(-\text{NH} \text{ \text{SO}_3\text{H})$	-	-	+	+	+	+	+	+

++ = growth from inoculum of 10^7 cells/ml

- = no growth

cells The effect is not due to any alteration of the assimilation process which has the same properties as before, nor is it due to micelles of dye taking glutamic acid mechanically into the cell since exactly

the same results are obtained by submitting the cells to the dye and washing them free from excess dye prior to the assimilation experiment. Optimum effects are obtained with an amount of dye which bears the same relation to the quantity of cells used, as the amount of dye necessary to inhibit growth bears to the inoculum in the growth tests. The difference between the levels with and without dye may

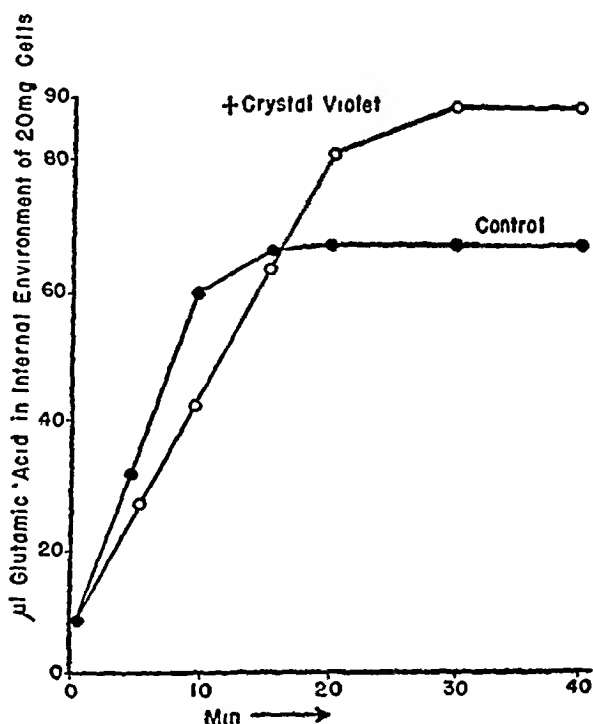


FIG 1 LECTURE II Effect of crystal violet (1/10,000) on assimilation of glutamic acid into the internal environment of deficient *Strep faecalis* cells. Cells incubated at 37° in salt solution containing 0.5% glucose and glutamic acid (200 μl/ml)

vary widely with the age of the cells, with cells harvested from a 6-8 hr culture the dye-treated cells may attain an internal level some 200-300% higher than the untreated cells (12)

An increase in the level attained during assimilation should indicate an inhibition of the metabolism of glutamic acid within the cell. This can be checked as follows: deficient cells are suspended in a solution of glutamic acid and glucose and incubated for an hour at 37° as usual,

but the glutamic acid content of the cells and the supernatant is accurately assayed both before and after the incubation. If this is done we find that there is no loss of glutamic acid during the experiment if glucose is not present, i.e. if no assimilation takes place, but that, if glucose is present, then the amount of glutamic acid disappearing from the external environment is markedly greater than that which appears within the cells. In the case quoted, (12), of a total amount of approx. 1800 μ l glutamic acid, 568 μ l disappeared during the assimilation process. It can be presumed that this glutamic acid has been metabolised in such a way that it can no longer be estimated by the decarboxylase method. However if the experiment is carried out in the presence of Crystal Violet and glucose, then the amount of glutamic acid which disappears during the assimilation is little greater than the experimental error.

The results can be checked by taking *Strep faecalis* cells which contain a large amount of free glutamic acid and suspending them in amino-acid free media, in the absence of glucose no change occurs, in the presence of glucose, glutamic acid passes out of the cell as described before but assays on (cells + supernatant) show that there is a disappearance, in the case quoted (12), of 107 μ l of a total of 250 μ l during the migration. Again if the experiment is repeated in the presence of Crystal Violet, the migration still occurs in the presence of glucose but the amount disappearing from the internal environment is equal, within experimental error, to that appearing in the external medium.

It seems fairly definite that there is a metabolism of glutamic acid within the cell and that this is inhibited by Crystal Violet with the result that there is a rise of free glutamic acid within the cell.

You will remember that the triphenylmethane dyes vary in antibacterial activity with structure, we can determine their comparative effects on this metabolism by studying their effects on the level achieved during assimilation. Table 2 summarises various properties of the dyes using Crystal Violet as the standard for comparison. It can be seen clearly that the effect on metabolism is closely parallel to the antibacterial action as judged by the inhibition of growth, suggesting that the latter is related to the former. It has often been suggested, since the biologically active form is the cation that the dyes vary in

efficiency due to varying ionisation with structure, it is certainly true that these dyes are all more effective in alkaline than acid media while acidic derivatives have no activity and are not fixed by the bacterial cells. However, it can be seen that there is no correlation between the effect on the intact cell and the ionisation constant, and that the most basic of all the series, methyl green, is devoid of activity

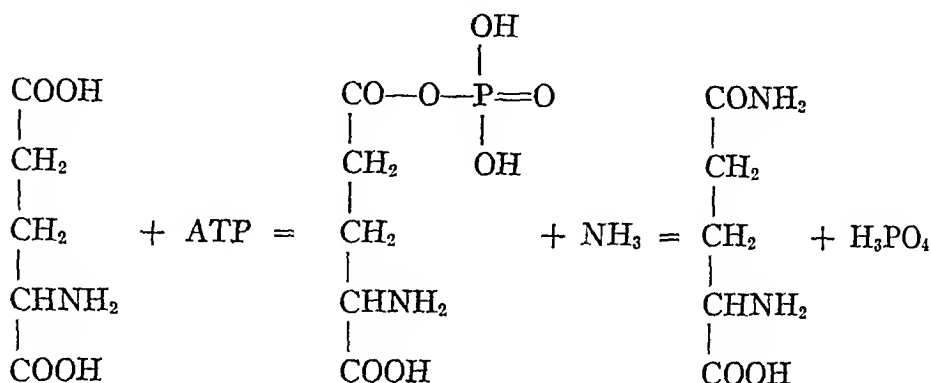
TABLE 2 (LECTURE II)
Properties of Triphenylmethane Dyes

NAME	SUBSTITUENT GROUPS	INHIBITION INDEX CV = 100	ASSIMILA TION INDEX CV = 100	PARTITION COEFFICIENT ISOBUT / WATER CV = 100	pKa
p-Rosaniline	$-\text{NH}_2, -\text{NH}_2, -\text{NH}_2$	10	4	16	7.57
Fuchsin	$-\text{NH}_2, -\text{NH}_2$	10	25	19	
	$-\text{NH}_2, -\text{CH}_3$				
Methyl Violet	$-\text{N}(\text{CH}_3)_2$	60	85	82	
	$-\text{N}(\text{CH}_3)_2$				
	$-\text{NHCH}_3$				
Crystal Violet	$-\text{N}(\text{CH}_3)_2$	100	100	100	9.36
	$-\text{N}(\text{CH}_3)_2$				
	$-\text{N}(\text{CH}_3)_2$				
Malachite Green	$-\text{N}(\text{CH}_3)_2$	140	120	164	6.90
	$-\text{N}(\text{CH}_3)_2$				
Brilliant Green	$-\text{N}(\text{C}_2\text{H}_5)_2$	200	143	632	7.90
	$-\text{N}(\text{C}_2\text{H}_5)_2$				
Methyl Green	$-\text{N}(\text{CH}_3)_2$	<1	0	0.25	
	$-\text{N}(\text{CH}_3)_2$				
	$-\text{N}(\text{CH}_3)_3\text{Cl}$				

on growth or metabolism. When we remember that the metabolism is intracellular and that there is a cell-wall which contains a high proportion of lipid, it seems more probable that the activity of the dyes will depend upon their ability to penetrate the cell-wall. As an indication of the lipid-solubility of the dyes, their partition-coefficient between isobutanol and water was determined, it can be seen that there is a close correlation between the partition in favour of isobutanol and the biological activity.

The next question which arises is—what is the metabolism of glutamic acid which is inhibited by these dyes within the cell? As we have already seen, we have a wide variety of reactions to test. Many Gram-negative organisms possess a glutamic dehydrogenase which oxidises the substrate to alpha-ketoglutaric acid. The Gram-positive cocci however do not appear to possess this enzyme, if they do its activity is so weak that it comes within the experimental error of the test method. A variety of organisms possess glutamic decarboxylase but this enzyme has not yet been found in either *Strep faecalis* or *Staph aureus*. Lichstein & Cohen (1) have demonstrated that these organisms, and many others, possess a highly active transaminase catalysing transamination between glutamic acid and oxalacetic acid, the enzyme can be obtained in a cell-free state from *Strep faecalis* and Prof Gunsalus kindly gave us some to test with the triphenyl-methane dyes. The enzyme showed little sensitivity to Crystal Violet and significant inhibition was obtained with concentrations of dye 100-1000 times those used in our metabolism tests. It is of course difficult to judge what the inhibitory concentration of the dye within the cell is, but this seems well outside reasonable limits. The growing bacteria must, of course, condense glutamic acid into protein and into peptide structures of the folic acid and glutathione types, if this were the metabolism involved here it should be possible to recover the disappearing glutamic acid by acid hydrolysis. The assimilation experiments with dyes are carried out with washed suspensions and we can find no evidence of protein formation in such preparations. Acid hydrolysis does not lead to a recovery of the "lost" glutamic acid so it is unlikely that protein formation is the reaction inhibited in these experiments. That appeared to be the sum total of testable hypotheses at the time this work was originally carried out and we had to fall back on the old cliché of "metabolic pool" to explain the lost glutamic acid. However during 1947 Elliott (13) in Cambridge, and Speck (14) working independently, discovered that there is another type of glutamic acid metabolism involving phosphorylation. When Krebs (15) was working on the synthesis and breakdown of glutamine in mammalian tissues, he found that synthesis will occur only if energy is supplied by some activity such as glycolysis. Elliott now found that glutamine synthesis will occur if tissue extracts are incubated

with glutamic acid and ammonia in the presence of adenosine-tri-phosphate and that inorganic phosphate equivalent to the glutamine is liberated during the synthesis. He has isolated the enzyme and it appears probable that the first step in the synthesis is a phosphorylation of glutamic acid in the gamma-position



In the presence of ammonia an interchange takes place between the phosphate group and ammonia to give glutamine with release of inorganic phosphate. Ammonia is not however the only possible reactant, in the presence of hydroxylamine, the hydroxamic acid is formed and this can be estimated colorimetrically. We have not yet had time to explore the full possibilities of this reaction although it may play a part in the formation of any γ -glutamyl compound. Ellhott discovered the enzyme first in brain tissue and we then proceeded to look for its presence in bacteria. The work has not yet progressed very far but we have found a highly active enzyme in *Staph aureus*. Washed suspensions of the organism have no activity but if the cells are mechanically disintegrated the enzyme is released in an active state. The bacterial enzyme has two interesting properties first, it is competitively inhibited by methionine sulfoxide and so provides a further example of competitive inhibition by metabolite analogues (16). Methionine sulfoxide was first reported by Waelsch *et al* (17) to act as a competitive inhibitor of the utilisation of glutamic acid by *L. arabinosus*, since it was ineffective in the presence of glutamine these workers suggested that it inhibited the formation of glutamine from glutamic acid and this suggestion has now been proved with the cell-free system. The formation of the

hydroxamic acid is 72% inhibited by M/90 methionine sulfoxide if the concentration of glutamic acid is M/30 while a four fold increase in the glutamic acid concentration results in a 20% decrease in the inhibition by the same concentration of methionine sulfoxide

Secondly, the enzyme system is sensitive to Crystal Violet The activity of the cell-free enzyme system was completely inhibited by a concentration of M/4,000 Crystal Violet (or 1/10,000 dilution) which is the order of concentration necessary to inhibit metabolism in the experiments carried out with washed suspensions The inhibitory concentration is critical as a further dilution to M/40,000 renders the dye ineffective as inhibitor It will be of interest to determine the relative inhibitory activities of the various triphenyl-methane dyes on this cell-free system but we have not yet been able to do this as practical difficulties arise when the hydroxamic acid reaction is carried out in the presence of some of the dyes and we are awaiting purification of the enzyme before proceeding further with the quantitative aspects of the dye inhibition We do know that p-Rosaniline is a much less effective inhibitor than Crystal Violet

We still cannot state categorically that this enzyme which carries out a phosphorylation of glutamic acid is necessarily the metabolic system which is inhibited by the dyes in the intact cells and whose inhibition results in a cessation of growth All we can say is that this enzyme is one involved in the intracellular metabolism of glutamic acid and is sensitive to Crystal Violet in concentrations which probably approximate to those holding within the cells exposed to the dye, also that none of the other metabolic systems tested that might possibly be involved are sensitive to Crystal Violet in concentrations of this order

The work just described concerning the effect of dyes on assimilation and metabolism of glutamic acid proves the truth of the suggestion that a rising level inside the cell during assimilation may be due to inhibition of intracellular metabolism If we study the ability of *Strep faecalis* cells to assimilate glutamic acid with and without Crystal Violet at various phases of their growth, we get curves such as those shown in fig 2 The top curve represents the level obtained in 100 mg cells harvested at various times during growth when allowed to assimilate glutamic acid to saturation in the presence of Crystal

Violet The experiments are carried out with washed cells and the level obtained must represent the assimilation achieved in the absence of intracellular metabolism. It can be seen then that the rate of assimilation is roughly constant throughout the growth period but falls rapidly after cell-division has ceased. This emphasises the necessity for using cells harvested during the growth period for experiments of this nature. The second curve represents the level obtained

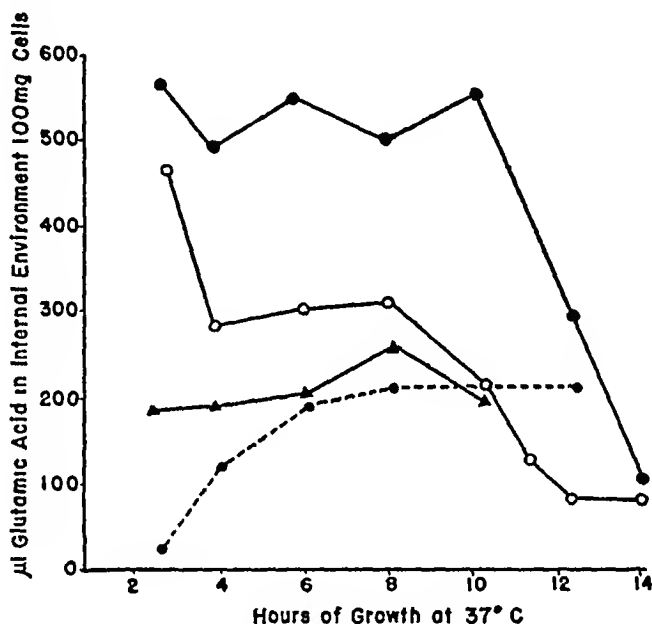


FIG 2 LECTURE II Internal concentration of glutamic acid in *Strep faecalis* cells at various ages of culture. Curve 1 = accumulation within growing cells, 2 = accumulation within resting cells, 3 = accumulation within resting cells treated with crystal violet, Curve 4 = growth curve

with the cells in the absence of dye, in each case the level is determined by the balance between the rate of assimilation and the rate of metabolism, so the difference between the two curves is a measure of the rate of dye-inhibited metabolism. This appears to be maximal during the linear phase of growth and to fall almost to nil soon after the cessation of cell-growth—as a routine measure we have used 6 hr cultures for all these experiments.

In these experiments the cells have been grown in a medium containing about 200 μ l free glutamic acid/ml, made into washed sus-

pension and then allowed to reach assimilation equilibrium in a medium containing 200 μ l free glutamic acid/ml. If we estimate the glutamic acid content of the cells directly on harvesting we get curve 1 which meets curve 2 at the time when active cell growth finishes. The lower level in growing cells as compared with washed cells equilibrated with the same external concentration of glutamic acid would lead us to expect that some form of internal metabolism is taking place in the growing cells over and above that in resting cells. Remember-

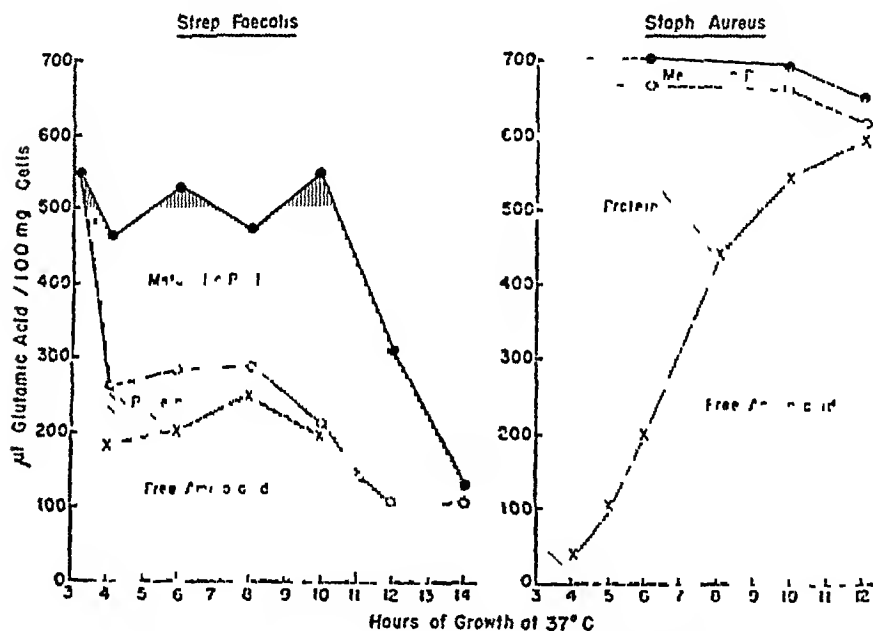


FIG 3 LECTURE II UTILISATION OF GLUTAMIC ACID

ing again that the levels indicated in this diagram represent a balance of rates it can be seen that this form of metabolism proceeds at its greatest rate in early cultures and ceases when growth ceases.

Fig 3 compares the types of curves obtained for *Strep faecalis* and *Staph aureus* and it can be seen that there are large quantitative differences between the two organisms. In *Staph aureus* the rate of metabolism in washed cells is small compared with the rate of assimilation but the metabolism in growing cells is more striking than that in *Strep faecalis*. I shall show in a minute that this new form of metabolism is protein synthesis and fig 3 shows that, in *Staph aureus*, the

rate of protein synthesis is nearly as great as the rate of assimilation in cells harvested during the early stages of growth but that, as growth continues, the rate of protein synthesis falls off in comparison with the rate of assimilation so that free glutamic acid steadily accumulates within the cells. At the end of growth, protein synthesis ceases and nearly all the assimilated glutamic acid appears in the free state within the cell.

The decarboxylase technique estimates only the free form of the amino-acid so, if peptide condensation takes place, the assay of glutamic acid will decrease. Acid hydrolysis will however recover the free amino-acid again and render it estimable by the enzyme. Table 3

TABLE 3 (LECTURE II)

Free and Combined Glutamic acid content of growing Staphylococcus aureus cells

AGE OF CULTURE	GROWTH (MG DRY WEIGHT OF CELLS/ML)	GLUTAMIC ACID (μ l /100 MG CELLS)			
		Free	Total	Combined	Ratio Combined/Free
<i>hr</i>					
3	0.07	160	1050	890	5.58
4	0.127	188	981	793	4.23
4½	0.164	215	1083	868	4.04
5	0.179	289	1116	827	2.86
6	0.232	371	1147	776	2.09
7	0.264	405	1134	729	1.80
8½	0.302	405	1003	589	1.48

shows the results of glutamic acid assays on cells before and after acid hydrolysis. When results are expressed in terms of 100 mg cells, it can be seen that the total glutamic acid content is constant whatever the age of the culture but that the free glutamic acid content rises as the culture approaches the cessation of growth—consequently the combined glutamic acid decreases with increasing age of culture.

Referring back to fig. 3 it can be seen that the difference between curves 2 and 1 can be accounted for in terms of combined glutamic acid. In the growing cell the level of free glutamic acid is lower than that in the resting cell, the difference in the two levels is due to the glutamic acid entering into protein synthesis and can be accounted for by recovery on acid hydrolysis. This means, incidentally, that

the reaction being studied in these curves is a condensation of glutamic acid into protein as glutamic acid and not after transformation into other amino-acids. The changing conditions within the cell are emphasised by the changing ratio of total/free glutamic acid with aging of the culture (18)

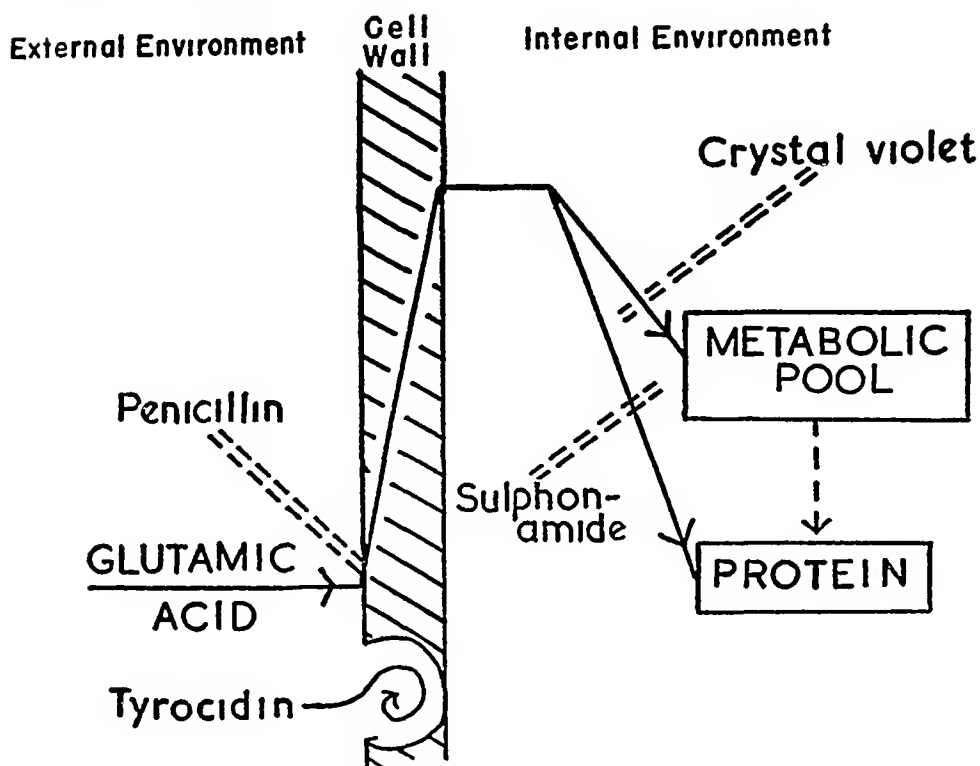


FIG 4 LECTURE II ASSIMILATION OF GLUTAMIC ACID

Fig 4 summarises the position we have reached at this moment. Glutamic acid enters the cell as a result of an active process requiring glycolysis or other exergonic metabolism on the part of the cell to carry the amino-acid into the cell against the concentration gradient. The level within the cell is determined by the balance between the rate at which the amino-acid crosses the cell-wall and the rate at which it is metabolised within the cell. By a study of the effects of growth and of dyes, we have shown that glutamic acid undergoes at least two forms of metabolism within the cell, first, a direct condensa-

tion to protein within the growing cell at a rate which varies with the age of the culture and second, a form of metabolism which is inhibited by dyes of the triphenylmethane series and which probably involves, as the first stage, a phosphorylation reaction. It is obvious that the assimilation process followed by concentration in the internal environment provides the cell with a reservoir of free amino-acid for its anabolic processes.

I want to turn now to the effect of sulphonamides on the utilisation of glutamic acid. Sulphathiazole in saturated solution has no significant effect on the level achieved within the cell during assimilation of glutamic acid by washed suspensions of either *Strep faecalis* or *Staph*

TABLE 4 (LECTURE II)

Effect of Sulphathiazole on accumulation of glutamic acid in internal environment of growing Staphylococcus aureus

All cultures inoculated at time 0 with same strain of *Staph aureus* and incubated at 37°C. Sulphathiazole added as below at 1 hr, all cells harvested at 4½ hr and washed before assay.

SULPHATHIAZOLE CONTENT OF GROWTH MEDIUM (MG /100 ML)	GROWTH AT HARVESTING (MG DRY WT CELLS/100 ML)	GLUTAMIC ACID IN INTERNAL ENVIRONMENT (μL /100 MG CELLS)
0	0 146	198
1	0 135	280
10	0 121	306
100	0 102	325

aureus, nor has it any effect on the glutamine-synthesising enzyme. As many of you will know, it is difficult to demonstrate an action of sulphonamides on *Staphylococci* growing in peptone or amino-acid media as the peptone and certain amino-acids are antisulphonamide in action. However it is possible to demonstrate a significant slowing of the rate of growth in such media by high concentrations of sulphathiazole. If we add sulphathiazole to cultures of *Staph aureus* when these are passing out of the lag phase of growth, we get a slowing down of growth and table 4 shows that the glutamic acid level within cells harvested a few hours later is significantly higher than that in cells from comparable cultures without sulphathiazole, further, the magnitude of the increase is related to the concentration of sulphathiazole in the medium. Fig 5 shows the same effect when samples are taken at

various times during the growth period and the rise of internal level in the early stages of growth compared with that in the culture without sulphathiazole can be clearly seen. The rise of level within the cell is, as we know, associated with inhibition of intracellular metabolism. Further we know that the difference between the level of free glutamic acid within the growing cell in early and late cultures, is due to the

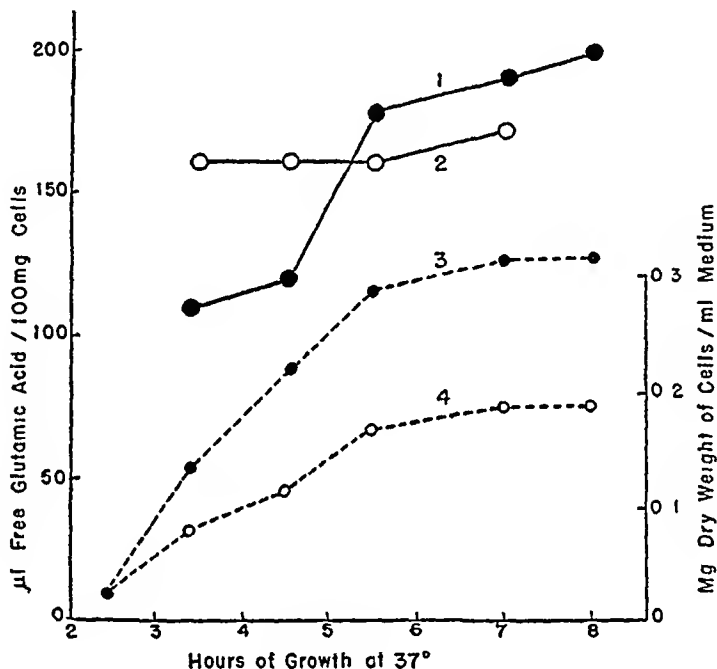


FIG 5 LECTURE II EFFECT OF SULPHATHIAZOLE ON ACCUMULATION OF FREE GLUTAMIC ACID IN GROWING STAPH AUREUS

Curves 1 and 3 Glutamic acid accumulation and growth in absence sulphathiazole. Curves 2 and 4 in presence of 100 mg % sulphathiazole

greater rate of protein condensation occurring in the early cultures. It follows then that the effect of sulphathiazole is to inhibit the condensation of glutamic acid into protein and it can be shown that the ratio of total/free glutamic acid in the sulphathiazole-treated cells is much lower than usual for cultures of this age and approximates to that attained in normal cells at the end of growth.

It is not possible to state categorically that sulphathiazole interferes with protein synthesis itself from these results as any reaction which

affected the rate of protein synthesis would have a similar result. I believe it is accepted fairly generally that one primary reaction of sulphonamides is to inhibit the metabolism of p-aminobenzoic acid, as first demonstrated by Woods (19). More recently we have seen that p-aminobenzoic acid forms part of the structure of pteric acid and the folic acid complex, and Lampen & Jones (20) and also Woods [private communication] have shown that the synthesis of pteroyl-glutamic acid from p-aminobenzoic acid is competitively inhibited by sulphonamides. It seems probable (21) that p-aminobenzoic acid may play rôles other than that involved as part of the folic acid complex in bacteria and a number of studies (22, 23) have indicated that either p-aminobenzoic acid or folic acid is involved in the synthesis of some amino-acids, methionine in particular. I showed just now that the reaction whose rate we study in these assimilation curves is that of the condensation of glutamic acid into protein, this necessarily involves condensation of other amino-acids so interference with the synthesis of any other amino-acid involved in the protein synthesis would necessarily result in a slowing of the rate of glutamic acid condensation. Consequently these results do not necessarily involve any new mechanism of sulphonamide inhibition but merely demonstrate one of the consequences of such inhibition.

However one of the amino-acids which has been reported to have an antisulphonamide action is glutamic acid and in the course of these studies we received the impression that the higher the glutamic acid content of the cell, the less susceptible to sulphonamide inhibition it became. We confirmed this in the case of a strain which is fairly sensitive to sulphathiazole in the following manner: a nutrient medium was prepared from casein-hydrolysate treated to remove vitamins and which was incubated with glutamic decarboxylase until all the glutamic acid was destroyed, this was then made up with a suitable salt mixture, glucose, nicotinamide and thiamine. Graded amounts of glutamic acid were then added and the growth in the presence of sulphathiazole tested for each concentration. Fig 6 shows that the presence of glutamic acid increases the sulphathiazole resistance, if we compare the effect of external glutamic acid concentration on internal concentration with its effect on sulphathiazole resistance, we can see that there is a correlation between the two curves which suggests that

the sulphathiazole resistance is determined by the internal concentration of glutamic acid rather than the external concentration

Fig 7 shows the accumulation of free glutamic acid within the cells of four strains of *Staph aureus* growing under identical conditions in the same casein-digest medium. The curves are all of the type with which we are now familiar, showing a decreasing rate of protein synthesis and consequent accumulation of free glutamic acid throughout

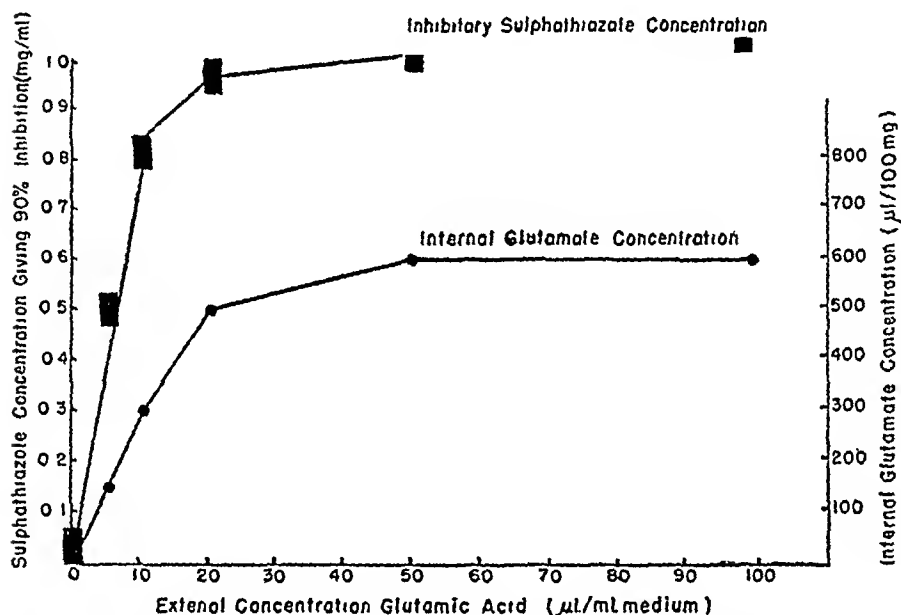


FIG 6 LECTURE II Effect of internal concentration of Glutamic acid on (a) internal concentration of glutamic acid and (b) concentration of sulphathiazole necessary to give 90% inhibition of growth

the growth period. The four organisms differ in their sulphathiazole resistance. Duncan and 156 are resistant to 10 mg % in growth inhibition tests, 6773 is sensitive to 1 mg % and the resistance of 1560 is intermediate between the two, somewhere around 5 mg %. These curves were determined in the hope that they would reveal some difference in the glutamic acid assimilation which could be correlated with the sulphathiazole resistances. It is immediately obvious that it is not the rate of assimilation itself which is involved; a measure of this is obtained by the level at the end of growth when protein synthesis

is over The rate of protein synthesis is determined by the difference between this final level (strictly speaking, the final level determined in the presence of an inhibitor such as Crystal Violet but the difference does not affect the argument in these cases) and the level at each age of culture, even in young cultures when the rate of protein condensation is greatest, there is no correlation between this rate and

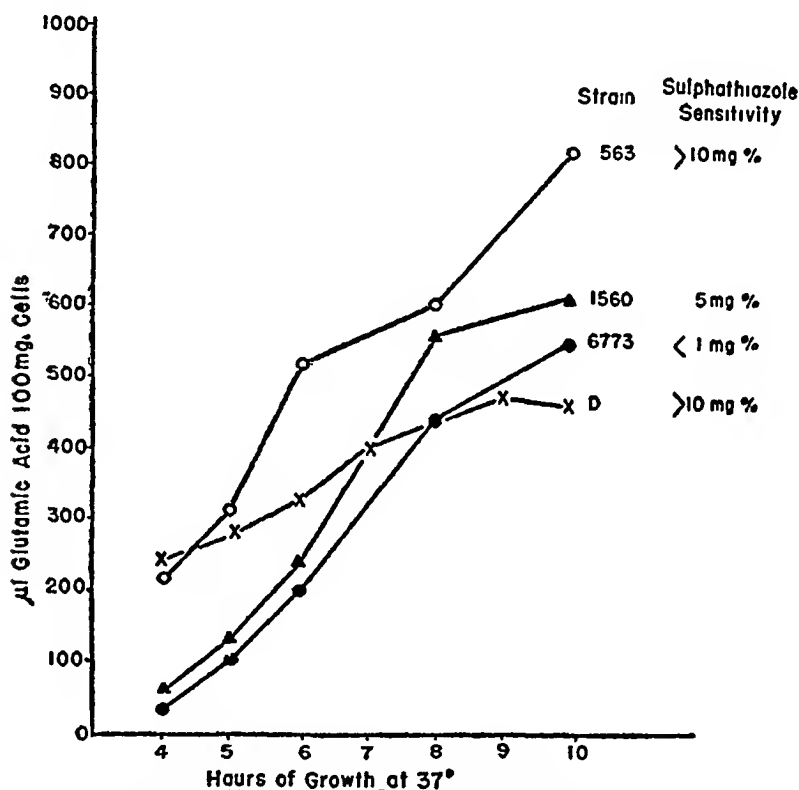


FIG 7 LECTURE II Accumulation during growth of free glutamic acid in internal environment of 4 strains of *Staph aureus* of differing sulphathiazole sensitivity

sulphathiazole resistance There is however one quantity which shows correlation with sulphathiazole resistance and that is the height of the level of free glutamic acid in the young cells, the resistant organisms have high levels at about the same value, there is very little free glutamic acid in the most sensitive strain, and the intermediate organism shows an intermediate level It must again be emphasised that this

level represents the balance of the rate of assimilation over the rates of internal metabolism and also represents the "driving head" of glutamic acid within the cell at this age of culture. As such it will effect by mass action any reaction involving glutamic acid as one of the reactants.

It is tempting to wonder whether it is the synthesis of pteroyl-glutamic acid again which is involved in the relation between glutamic acid and sulphathiazole resistance. This point is now being investigated but insufficient evidence has been obtained to enable us to make any definite statement.

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LECTURE 3

THE NATURE OF PENICILLIN SENSITIVITY IN STAPHYLOCOCCUS AUREUS

The studies so far described on the assimilation and utilisation of glutamic acid by Gram-positive bacteria may be summarised in this manner. Glutamic acid enters the cell as a result of an active process on the part of the cell and undergoes marked concentration during its passage across the cell-wall. The maximum degree of concentration attainable across the cell-wall varies with strain and species, but the level of free glutamic acid measured within the cell depends upon the balance between the rate at which the amino-acid is assimilated into the cell and the rate at which it is metabolised within the cell. We have been able to identify a number of reactions going on inside the cell and all of these make it clear that the free glutamic acid accumulated within the cell acts as the reservoir upon which the cell draws for its anabolic reactions. The various inhibitors that we have studied in the last lecture had their actions on these intracellular reactions and now, in this last lecture, I wish to turn to an inhibitor which has an action on the assimilation process rather than on the intracellular utilisation.

The story of the discovery of penicillin is too well-known to need recounting, and rates as a monument to scientific international co-operation and as the most beneficial accident ever to happen in a bacteriological laboratory. Penicillin is excreted by the mould *Penicillium notatum* and has very powerful bactericidal action on a variety of bacterial species, mostly belonging to the Gram-positive group although the Gram-negative *Neisseria* are amongst the most susceptible organisms. Although a vast literature has accumulated concerning the production, chemistry, conditions of action and clinical applications of penicillin, there has been comparatively little published on the mechanism of penicillin inhibition. Chan & Duthie (1) showed that whereas penicillin has no effect on the respiration of resting cells, its addition to growing cultures of *Staph aureus* gives rise to a progressive inhibition of respiration until this eventually ceases altogether. The fact that penicillin will act only on cells in a growing state was clearly demonstrated by the finding that a bacteriostatic agent such as helvolic acid will protect the cells against penicillin.

whereas sulphonamides, which do not exert their action until the cells have undergone several divisions, have no such protective action. These workers also found that if penicillin is added to a growing culture in the lag or logarithmic phase then growth continues to the extent of not more than one division per cell, after which cell-division ceases although increase in cell size without division may then take place. Cessation of growth is followed by a fall in both viable and total counts and general lysis may take place after a period of some hours. Hirsch (2) working independently at the same time found similar effects upon the respiration of *Staph aureus*—a progressive falling-off in the respiration after penicillin addition to growing cells but not to resting cells—and suggested that the action of penicillin is to produce a degenerative change which results in the production of a sterile generation of cells. Krampitz & Werkman (3) have shown that high concentrations of penicillin will inhibit the dissimilation of ribonucleic acid added to suspensions of *Staph aureus* and will also inhibit the endogenous breakdown of ribonucleates while having no effect on the metabolism of desoxyribonucleates.

Fig 1 shows the effect of the addition of various concentrations of penicillin to growing cultures of *Staph aureus*. If the increase in bacterial substance is followed turbidimetrically we find that growth continues at the normal rate for about 30 mins after the penicillin addition and the rate then falls off and stops after a time which depends to a certain extent on the concentration of penicillin used. The addition of 10 units penicillin/ml brings growth to a stop within 90 min whereas 0.1 units/ml takes $2\frac{1}{2}$ –3 hr to become completely effective. Since we require a reasonable quantity of organism for estimations of the nature discussed in these lectures we cannot work with organisms harvested during the very early phases of growth and, in general, it has been the custom to add penicillin at about the third hour of growth and harvest at the 4th or 5th hour. Fig 2 shows what happens to the accumulation of glutamic acid within the cells of a culture of *Staph aureus* grown in a casein-digest medium. In the absence of penicillin, the free glutamic acid content of the cells shows a steady increase throughout the growth period, as shown in the last lecture. If penicillin is added to the culture at the 4th hour of growth, the accumulation of free glutamic acid continues almost at the normal rate for

approximately an hour and the level then falls dramatically, finally coming to a new steady value at about the time that growth is completely inhibited

The work we have discussed so far has demonstrated that the level of free glutamic acid within the cell is determined by the balance between

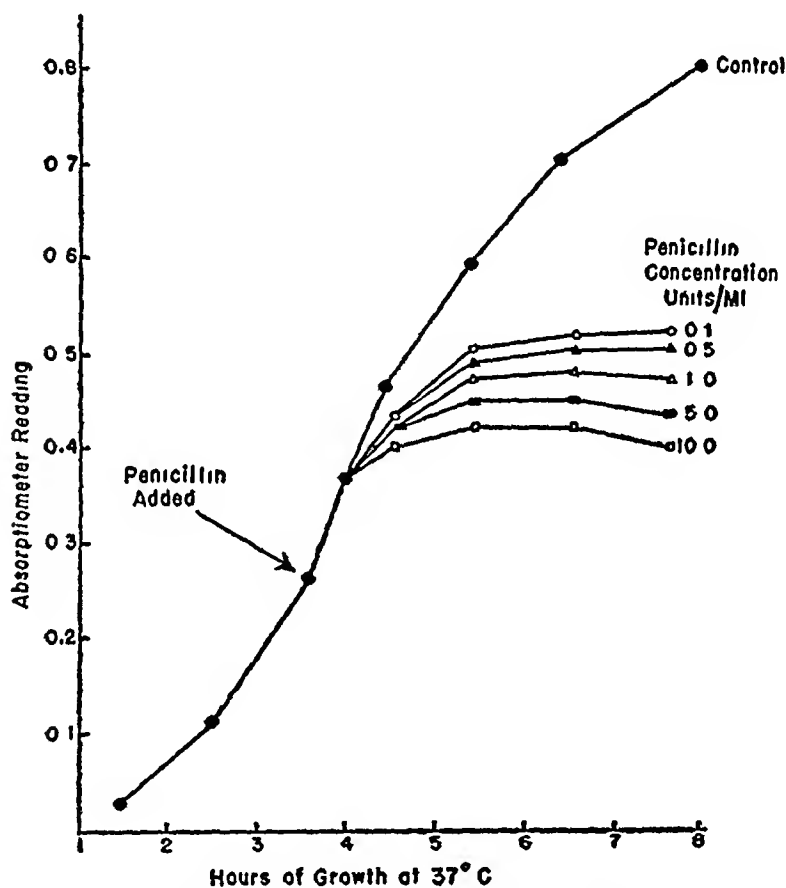


FIG 1 LECTURE III EFFECT OF ADDITION OF PENICILLIN TO GROWING CULTURES OF STAPH AUREUS

the rate of assimilation and the rate of internal metabolism. The addition of penicillin to the culture is followed by a rapid falling of the level within the cells, in agreement with our hypothesis it should therefore follow that penicillin either promotes an increase in the rate of internal metabolism or, alternatively, produces an inhibition of the assimilation process. It is possible to investigate the latter suggestion

by the method outlined in the first lecture of this series if the organism is grown in a deficient medium, then incubated in a salt medium containing a comparatively high concentration of glutamic acid and

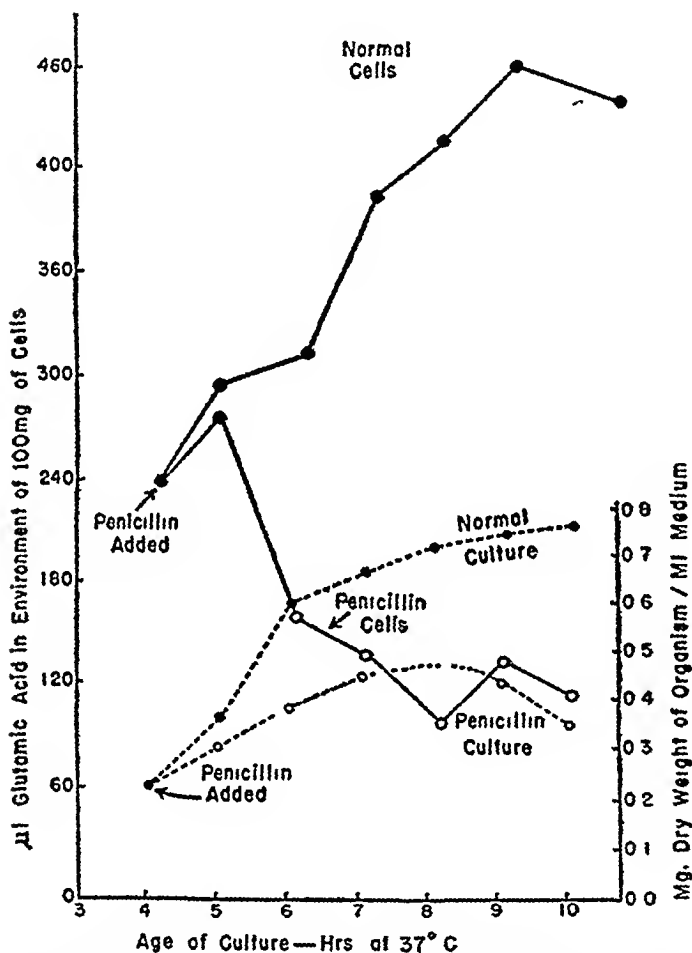


FIG 2 LECTURE III Effect of addition of penicillin to growing culture of *Staph aureus* on the internal accumulation of glutamic acid

glucose to provide energy for assimilation, the cells will take up glutamic acid until the new equilibrium between internal and external environments is established. The rise in the internal level under standard conditions of time and external environment can then be taken as a measure of the assimilation ability of the cells. Table 1

shows the amount of glutamic acid assimilated by 100 mg cells grown under various conditions and tested in this way. Penicillin was added to the culture at the 4th hour as usual and portions of the culture—together with similar portions from a control culture without penicillin—removed at intervals after the addition. In the control culture, the ability to assimilate glutamic acid is approximately constant (between 600 and 700 $\mu\text{l}/100\text{ mg}$) over the period examined. In the culture to which 10 units penicillin/ml has been added, the ability to assimilate is reduced to 14% of the control within 30 min, to 4%

TABLE 1 (LECTURE III)

Effect of the presence of penicillin during growth on the assimilation of glutamic acid by Staphylococcus aureus

Cells grown in a deficient medium and penicillin added in all cases after $3\frac{1}{2}$ hr growth at 37°C . Cells harvested at various times after the penicillin addition, incubated for 1 hr at 37°C in glutamic acid (200 $\mu\text{l}/\text{ml}$) and glucose (0.5%) and the increase in the internal glutamic acid content (expressed as per 100 mg cells) determined.

PENICILLIN CONCENTRATION (UNITS/ML MEDIUM)	GLUTAMIC ACID ASSIMILATED/100 MG CELLS				
	Time of harvesting after penicillin addition				
	30 min	1 hr	$1\frac{1}{2}$ hr	2 hr	3 hr
0	561	702	602	590	614
0.1	—	—	—	130	—
0.5	—	—	—	113	—
1.0	—	—	87	—	0
5.0	—	—	0	—	0
10.0	82	31	0	0	0

within 1 hr and after $1\frac{1}{2}$ hr assimilation is no longer possible. Smaller concentrations of penicillin have the same effect of blocking assimilation but take longer to become completely effective. If we compare the impairment of assimilation with the impairment of growth, we can see that there is a close correlation between the two effects (4).

It is important to discover to what extent this effect on assimilation is symptomatic of a general metabolic impairment. Table 2 shows the general metabolic activities of cells harvested from a culture which has grown in the presence of 10 units penicillin/ml for 90 min when compared with the activities of cells from a control culture without penicillin. The respiration is slightly below normal but the difference

is within experimental error—consequently the respiratory failure demonstrated by Chain & Duthie, and by Hirsch, has not yet set in. The oxidation of glucose and its fermentation are normal, consequently the impairment of assimilation is not a secondary effect due to impairment of the energy system. The assimilation of lysine is normal, you will remember that this process differs from the assimilation of glutamic acid in that it appears to be a purely physical process, the performance of which depends upon the integrity of the cell-wall, this result therefore demonstrates that no significant degree of lysis can have occurred in the cells at this stage. The assimilation of glutamic acid is completely blocked and the viable count is about 2%

TABLE 2 (LECTURE III)

Metabolic activities of normal Staphylococcus aureus cells and of cells grown in presence of penicillin

"Penicillin cells" grown for 90 min in medium containing 10 units penicillin/ml

	NORMAL CELLS	"PENICILLIN CELLS"
Respiration Q_{O_2}	21.5	19.6
Glucose oxidation Q_{O_2}	86.5	84.5
Glucose fermentation QCO_2 acid	96	108
Lysine assimilation (μ l/100 mg)	90	96
Glutamic assimilation (μ l/100 mg)	602	0
Comparative viable count	452	9

that of the control. It is important to notice that none of the reactions discussed, including glutamate assimilation, are affected in washed cells by the addition of penicillin even in concentrations of the order of 50 units/ml. The blocking of glutamic assimilation occurs only if the cells are grown for a short period in the presence of penicillin and so can again be correlated with the known antibiotic effects of penicillin on growing but not resting cells.

We now know of four effects produced by penicillin on growing cells: loss of viability, loss of the ability to assimilate glutamic acid, progressive respiratory failure, lysis. The results discussed so far show that assimilatory impairment precedes respiratory failure and lysis but it is not possible to state categorically whether it precedes loss of viability. The curves (fig. 2) showing the falling in glutamic level

after penicillin addition and the effect on growth as judged turbidimetrically show clearly that the level within the cell begins to fall before increase in cell-mass ceases. Further than that it is not safe to go at present.

We know from previous work that the glutamic acid within the cell acts as a reservoir for various forms of metabolism within the cell, it is important for us to know whether this internal metabolism is affected by penicillin. We can investigate this by a modification of the method used to show the inhibition of internal metabolism by dyes of the triphenylmethane series. Table 3 shows the results of such an experiment. Normal, "deficient" *Staph aureus* cells are in-

TABLE 3 (LECTURE III)

Effect of penicillin treatment on internal metabolism of glutamic acid in Staphylococcus aureus

	EXTERNAL ENVIRONMENT	INTERNAL ENVIRONMENT	CHANGE IN EXTERNAL ENVIRONMENT	CHANGE IN INTERNAL ENVIRONMENT	μ l GLUTAMIC ACID METABOLISED
Normal cells					
Initial	1586	690	-831	+370	461
Final	755	1060			
Penicillin- treated cells					
Initial	1646	579	-106	-263	363
Final	1540	315			

cubated in a known concentration of glutamic acid in the presence of glucose, and the change in the internal and external glutamic acid assayed after an hour, glutamic acid has left the external environment and passed into the cell with a consequent increase in the internal level, but a total of 461 μ l has been metabolised during the assimilation. An exactly similar experiment was carried out with cells of the same age but harvested from a culture to which 10 units penicillin/ml had been added 90 min before harvesting. We find that, in this case, 363 μ l glutamic acid have disappeared in the course of the incubation but a glance at the distribution between internal and external environments of this loss shows a very different picture from that obtained with the normal cells. In this case, very little glutamic acid has disappeared from the external environment—in comparison with the

831 μ l which disappeared in the control—and the metabolised glutamic acid has been withdrawn mainly from the internal environment. In other words, the internal metabolism of glutamic acid has proceeded normally in the penicillin-treated cells and has proceeded at the expense of the internal reservoir whereas, in the normal cells, any such internal utilisation is made good by assimilation from the external environment. In the penicillin-treated cells this is impossible since the passage of glutamic acid across the cell-wall is blocked.

The studies discussed so far were all carried out on a normal penicillin-sensitive strain of *Staph aureus*. The next question that arises is what happens with the so-called resistant strains? As you know it is a comparatively easy matter to produce penicillin-resistant strains from sensitive ones by a process of serial subcultivation in media containing steadily increasing concentrations of penicillin. The acquirement of resistance was studied by Demerec (5) who concluded that in every culture there occur mutants which have a somewhat greater resistance to penicillin than the bulk of the cells, when growth occurs in a concentration of penicillin which limits the growth of the bulk of the cells, then selective growth of the more resistant mutants takes place. The "training" process thus consists in gradual selection of more and more-resistant mutants and is presumably only limited by the ability of the organism to mutate in this sense. We took our strain of *Staph aureus* and proceeded to train it to penicillin in the usual way. When we had reached a resistance of 60 units/ml (an increase in resistance of some 600 times over that of the organism originally studied) we carried out tests on the blocking of glutamic assimilation in the same way, in this case the assimilation was unaffected by the addition to the medium of 1 or 10 units penicillin/ml but was completely blocked by the addition of 100 units/ml. Consequently an increase in the growth-resistance to penicillin was accompanied by an increase in the resistance of assimilation processes.

It seemed probable that some property of the assimilation process could be related to the penicillin resistance. Since we wished to study the assimilation process itself in the absence of complications due to internal metabolism, it was necessary to abolish this internal metabolism, to do this we took washed suspensions of the cells (in which we have previously shown that protein synthesis does not take place)

and treated them with amounts of Crystal Violet just sufficient to inhibit the residual internal metabolism. In these cells glutamic acid enters the cell until the internal concentration is in true equilibrium with the external concentration. Table 4 shows that the penicillin resistance of a number of various organisms cannot be correlated with their internal level of glutamic acid under standard conditions and in the absence of internal metabolism, the resistance cannot

TABLE 4 (LECTURE III)

Penicillin resistance and internal concentration of glutamic acid in a series of organisms

ORGANISM	STRAIN	PENICILLIN TEST		INTERNAL CONCENTRATION OF GLUTAMIC ACID $\mu\text{l}/100\text{ MG}$
		Growth Units/m	No Growth Units/ml	
<i>Staph aureus</i>	563	0.02	0.04	1165
<i>B. subtilis</i>	St	0.04	0.06	26
<i>Staph aureus</i>	209	0.05	0.10	560
<i>Staph aureus</i>	D	0.06	0.08	660
<i>Staph aureus</i>	6773	5.0	7.0	890
<i>Strep faecalis</i>	ST	6.0	8.0	534
<i>Staph aureus</i>	6773	9.0	15.0	880
		15	20	825
		60	70	750
		250	300	740
		2000	4000	705
		6000	—	0

then be correlated with the concentration gradient across the cell-wall (6)

Fig. 3 shows the effect of the external glutamic acid concentration on the internal concentration in the case of two organisms. The curves are somewhat different from those I showed in the first lecture and in which no attempt was made to control the internal metabolism—in the absence of internal metabolism the curves are sharper and flatter. In both cases the internal level is independent of the external concentration except for low values of the latter but it can be seen that the

concentration affected by *Staph aureus* at very low external levels is considerably greater than that effected by *Strep faecalis*—in other words, the slope of the *Staph aureus* curve is steeper than the slope for *Strep faecalis*. The slopes of these curves represent the affinity of the cells for the assimilation of glutamic acid and, in analogy with enzyme-substrate curves, we can take, as a measure of this affinity, the reciprocal of the external concentration required to produce an

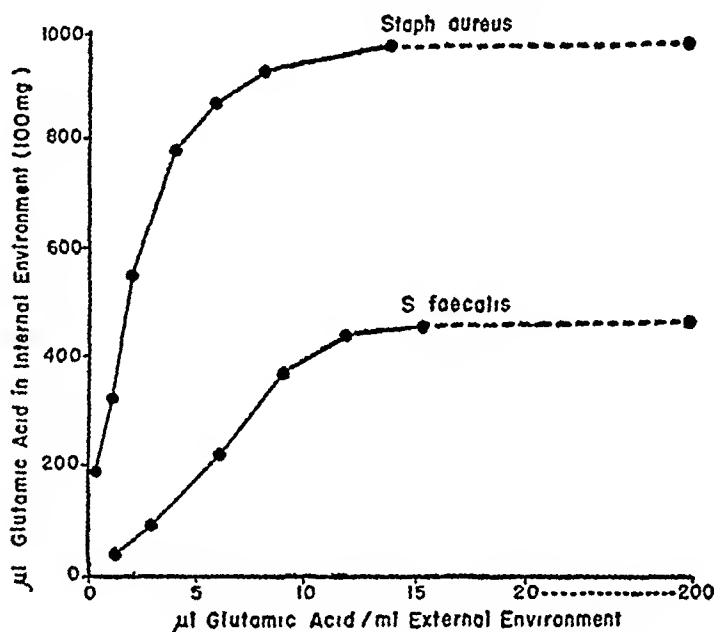


FIG 3 LECTURE III DEPENDENCE OF INTERNAL GLUTAMATE CONCENTRATION ON EXTERNAL CONCENTRATION

internal concentration equal to half that attained at saturation. In the case of *Staph aureus*, this external concentration is 1–2 μ l while for *Strep faecalis* it is 6–7 μ l. *Strep faecalis* is markedly more resistant to penicillin than *Staph aureus* and these curves gave us the idea of investigating the value of the “assimilation affinity” in organisms of differing penicillin resistance. Fig 4 shows a series of such results—the ordinates are expressed as % saturation of the internal environment so as to avoid difficulties due to the varying saturation level in various organisms. Of a series of organisms which had different penicillin-resistances on isolation, there is a correlation in

that the higher the resistance, the higher the assimilation constant (or the lower the assimilation affinity). The correlation was proved by taking the organism with resistance of 15 units/ml and training it by serial subcultivation in increasing concentrations of penicillin until it would eventually grow in 2000 units/ml. At intervals during the process the assimilation affinity was determined and the curves show that this steadily decreased as the resistance increased.

Fig 5 shows again the relation between values of the assimilation constant and the log of the penicillin resistance. Values for a moderately resistant *Strep faecalis* and for a sensitive *B subtilis* are in-

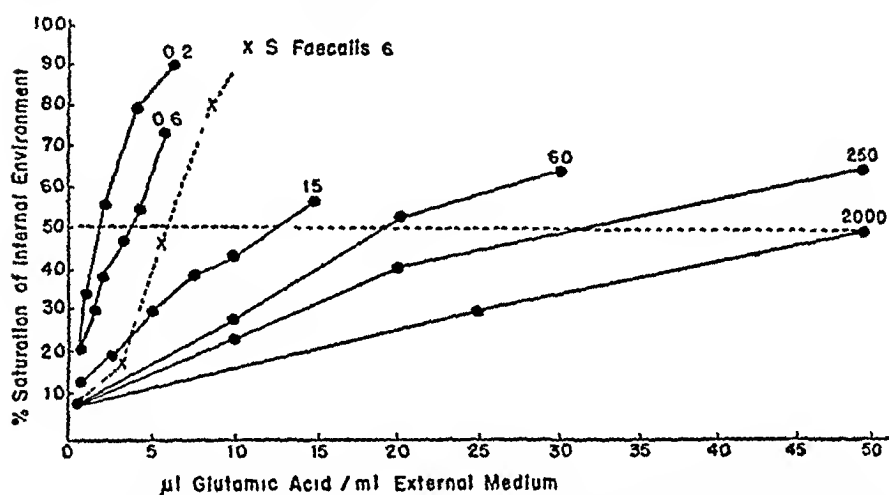


FIG 4 LECTURE III "ASSIMILATION AFFINITIES" (GLUTAMIC ACID) OF *STAPH AUREUS* OF DIFFERING PENICILLIN SENSITIVITIES

cluded and appear to fit in the general curve given by *Staph aureus* strains. As the resistance reaches high levels—of the order of 1000 units/ml—the assimilation affinity decreases very rapidly and it would appear that, if the penicillin resistance were pushed to any further heights, then the organism would no longer be able to assimilate glutamic acid effectively. As the organism is trained to increasing levels of penicillin resistance, the selection of resistant mutants involves also the selection of those mutants which depend less and less upon assimilation for their growth. We have already postulated that assimilation is a mechanism used by the organism to compensate for loss of synthetic power, if assimilation is impaired and the organism

still grows, it would seem from our postulate that it must do so by synthesis

The organism cultured from 2000 units/ml appears to be a normal *Staph aureus* from cultural and staining characteristics Between

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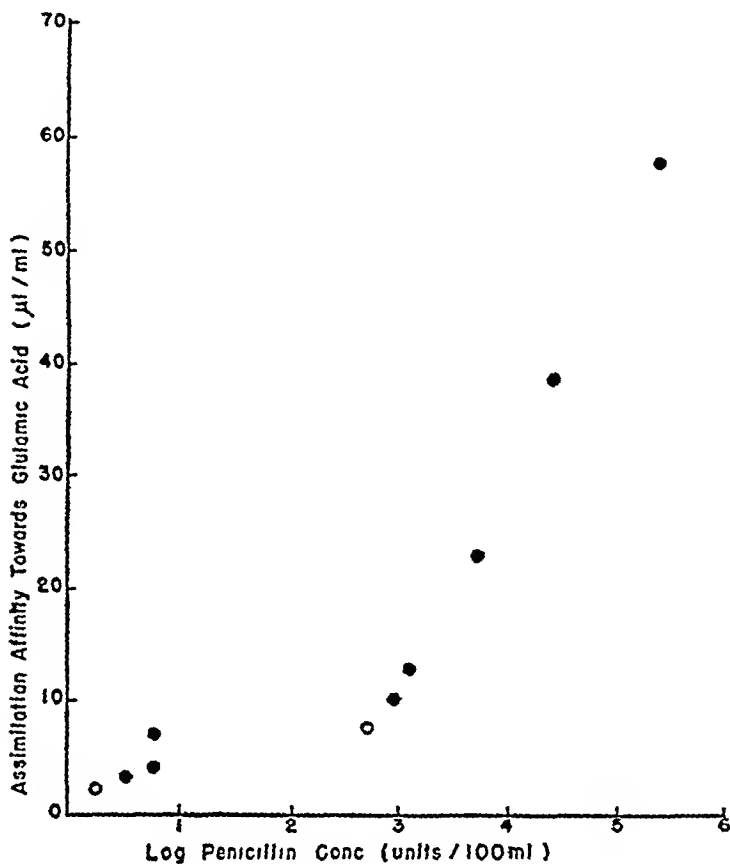


FIG 5 LECTURE III RELATION BETWEEN ASSIMILATION AFFINITY TOWARD GLUTAMIC ACID AND THE PENICILLIN RESISTANCE

2000 and 6000 units penicillin/ml it changes from a Gram-positive coccal organism to a Gram-negative bacillus displaying marked pleomorphism This spectacular change was first noted by Bellamy & Klimck (8) who were working independently on the penicillin resistance problem at the same time as we were carrying out our as-

simulation studies When their first note was published, I had taken my own organism to a level of 2000 units/ml and had stopped since I had obtained the information on assimilation affinity just described When I read the note of Bellamy & Klimek (7) I decided to force my organism, if possible, up to levels reported by these workers to see whether I could get the same changes as they described My organism was reluctant to increase its resistance above 2000 units, but by perseverance and large inocula it eventually grew first at 4000 and then at 6000 units/ml Plating of the 4000 unit culture gave a mixture of Gram-positive and Gram-negative forms while the 6000 units culture gave a pure culture of Gram-negative forms Dr Bellamy kindly sent me cultures of his resistant organism and I found that it had staining and cultural characteristics identical with mine—obtained from different parent strains, in different media and with different penicillin preparations Bellamy & Klimek (8) have published details of their training procedure and of the properties of their resistant organism—these properties are duplicated in the case of my resistant organism Both organisms are strict aerobes and have lost most of their fermentative powers with the exception of a feeble activity towards glucose Bellamy and I entered into an agreement for the study of these organisms in which I undertook to investigate their amino-acid metabolism

The general activities of all the organisms are all very similar and are essentially the same as those described by Hills (9) for *Staph aureus* In some cases, e g glycine, serine, proline and glutamic acid, the rates of attack by the resistant organisms are significantly lower than for the parent strains while in other cases, e g arginine and lysine, the rate of attack by the resistant strains is significantly higher The only case where the rate of deamination is significantly higher than the rate of oxidation is that of arginine, for all four organisms an anaerobic deamination of arginine with liberation of CO₂ can be demonstrated and it is highly probable that all four organisms possess arginine dihydrolase demonstrated by Hills (9) as the main ammonia-forming enzyme system of Gram-positive cocci All four organisms possess urease to much the same degree of activity A glance at the figures leaves little doubt but that we are dealing with genuine staphylococcal organisms and not with odd contaminants This is proved, as we

shall see later, by the fact that the resistant organisms will revert to give Gram-positive *Staph aureus*

If the organisms are subjected to the usual assimilation procedure, they show no internal glutamic acid at all under any condition of test. Here they conform to Taylor's law (10) that Gram-positive organisms contain free amino-acids in the internal environment whereas Gram-negative organisms do not. What about their abilities to synthesise amino acids?

Staph aureus strains are highly exacting towards amino-acids as demonstrated by Gladstone (11) and also require nicotinic acid and thiamine. We investigated the nutritional requirements of the various organisms by growing them first in Gladstone's synthetic medium and then testing their ability to grow in this medium from which the various components were omitted one at a time (12). Table 5 shows the results: the parent 6773 strain requires 10 amino-acids in addition to the two growth factors, the parent 209 requires 7. In the case of both resistant organisms the omission of any amino-acid has no appreciable effect on the growth, likewise the omission of nicotinic acid has no depressant effect. We made a jump and tested the growth of the organisms in a simple salt, glucose, thiamine and ammonia medium, neither parent strain will grow but either resistant organism will grow normally after a lag of a few hours—this lag being considerably reduced by the addition of cystine to the medium. In other words, the two resistant organisms can synthesise all their amino-acid requirements from ammonia and glucose and can also synthesise nicotinamide but not thiamine. The studies which we carried out on assimilation affinity suggested that increased penicillin resistance should select mutants which relied on synthesis rather than assimilation processes, pushed to its logical conclusion this should mean that penicillin training would eventually lead to the selection of mutants which can synthesise all their amino-acid requirements, this has now been realised experimentally.

We have studied the ability to synthesise amino-acids of organisms which represent opposite ends of the scale of sensitivity to penicillin. Can we determine any facts about the intermediate cases or obtain any further information on the relation between synthetic ability and penicillin resistance? Table 5 brings out a number of points bearing on

this problem Our parent sensitive strains 6773 and 209 are exacting to a variety of amino-acids, the Gram-negative derived organisms can synthesise all their amino-acid requirements and nicotinamide The case of the organism trained to a resistance of 2000 units penicillin/ml was the first studied, this organism is Gram-positive, a facultative

TABLE 5 (LECTURE III)
Relation between Penicillin Resistance and Synthetic Abilities

PARENT	MUTANTS SELECTED BY PENICILLIN TRAINING						
	6773	6773	6773	209	209 (PT)	209	209
Penicillin resistance (36 hr test)	5	2000	6000	0.05	6000	400* (reversion mutants)	1000*
Gram stain	+	+	-	+	-	+	+
Nicotinamide	+	+	-	+	-	-	-
Thiamine	+	+	+	+	+	+	+
Proline	(88)	(88)	-	+	-	(88)	(64)
Histidine	+	+	-	+	-	(64)	(64)
Valine	+	+	-	+	-	(88)	(88)
Glycine	+	(88)	-	+	-	(160)	+
Aspartic acid	+	(64)	-	+	-	(160)	(64)
Leucine	(40)	(40)	-	+	-	(40)	(40)
Cystine	+	+	-	+	-	+	+
Glutamic acid	(40)	-	-	-	-	(64)	(40)
Phenylalanine	(40)	(64)	-	-	-	(40)	(40)
Arginine	+	+	-	-	-	+	+

* Penicillin resistance increased as adaptation took place, after 84 hr resistance had increased to 800 and 1700 units/ml respect

+ = presence essential for growth

- = presence not essential for growth in same time as in complete medium

(88) = growth took place in absence after adaptation had occurred in 88 hr

anaerobe and normally pigmented, its synthetic abilities fall in a position intermediate between that of the parent 6773 and that of the Gram-negative derivative, in that it adapts to the absence of either glycine or aspartic acid whereas the parent 6773 cannot adapt to the absence of either, also it is non-exacting towards glutamic acid whereas the parent cannot grow in the absence of this amino-acid until adaptation has occurred after 40 hr

Gladstone (11) showed that strains of *Staph aureus* can be trained to dispense with amino-acids by the process of attrition involved in serial subcultivation in media containing progressively fewer amino-acids. We have applied the Gladstone technique to both our parent strains and have been able to train them to dispense with all amino-acids except histidine and cystine. Table 6 shows that this training process—which presumably involves selection of non-exacting mutants—results in a simultaneous increase in the penicillin resistance. Con-

TABLE 6 (LECTURE III)
Relation between Synthetic Abilities and Penicillin Resistance

	MUTANTS SELECTED BY NUTRITIONAL TRAINING			
	6773	6773	209	209
Parent strain				
Nutritional requirements				
Nicotinamide	+	+	+	+
Threonine	+	+	+	+
Proline	+	—	+	—
Histidine	+	+	+	+
Valine	+	—	+	—
Glycine	+	—	+	—
Aspartic acid	+	—	+	—
Leucine	+	—	+	—
Cystine	+	+	+	+
Glutamic acid	+	—	—	—
Phenylalanine	+	—	—	—
Arginine	+	—	—	—
Penicillin resistance (36 hr. test) units/ml	5.0	100	0.05	250

sequently there is a definite correlation between synthetic abilities and penicillin resistance in these strains.

Thirdly we have the interesting cases of two organisms obtained by reversion of the Gram-negative 209 derivative. While Bellamy and his co-workers were attempting to revert their organism to prove its staphylococcal nature, it several times underwent such reversion in our hands—as it were by accident! Between us we have worked out a technique which has enabled us to isolate several Gram-positive staphylococcal reverted mutants from the Gram-negative 209 PT organism. The reversion is due to chance mutation and our methods merely consist in a method whereby the growth of the reverted mutant

will be favoured once the reversion has occurred. The method consists in inoculating large inocula of Gram-negative organisms into a rich casein-digest medium and then two or three subcultivations into a medium containing either marmite or yeast extract in low concentration as sole nutrient. Table 5 shows the synthetic abilities of two such reversions from 209 PT, both reversions require a number of amino-acids although their requirements are less than those of the original parent 209. One very interesting fact is that both reversions have retained the power to synthesise nicotinamide whereas the original parent—together with most freshly isolated *Staph aureus* strains—has not this ability. The resistance towards penicillin is again of a high level but intermediate between that of 209 PT and 209. The strains are not stable in their requirements as they adapt fairly easily to the absence of most of the amino-acids—but not of arginine or cystine—and their resistance to penicillin also goes up from about 450 units/ml in a 30 hour incubation to over 1000 units in a 4 day incubation.

The results expressed in this table bring out two facts quite clearly first, that *Staph aureus* possesses a high rate of mutation and second, that there is a reciprocal relationship between the synthetic ability and the penicillin sensitivity of the strains obtained.

This all correlates very well with the original finding that penicillin blocks the assimilation process. We were restricted in our studies on assimilation to glutamic acid although it was always possible that the facts discovered with that amino-acid were symptomatic of changes in the assimilation process towards amino-acids in general. It is now certain that this must be the case, the penicillin-resistant organisms synthesise all their amino-acids and even moderately sensitive strains can dispense with glutamic acid. It is however definite that penicillin does not block the assimilation of lysine, which is, as you may remember, a physical process. Lysine is however not an essential amino-acid for the strains of *Staph aureus* studied.

We have seen that bacteria must grow either by assimilation or by synthesis of their constituent amino-acids, that some organisms have lost certain synthetic abilities and so are forced to assimilate, that many such obligate-assimilators effect a concentration of certain essential amino-acids within the internal environment prior to further metabolism and that all organisms which have acquired this ability

are also Gram-positive. We have seen that penicillin will block the assimilation process towards certain amino-acids, that training to penicillin-resistance results in the selection of less exacting-mutants, that there is a reciprocal relation between synthetic abilities and penicillin sensitivity, that penicillin-training pushed to its highest levels results in the selection of organisms non-exacting towards amino-acids and that such organisms are Gram-negative, that reversion mutations will occur to give organisms exacting towards amino-acids which are Gram-positive.

The Gram-positive cell is able to effect a concentration of certain amino-acids within the internal environment, a glance at the analytical figures at our disposal shows that the relative concentrations of free amino-acids within the cell are very roughly those of the relative proportions of the combined amino-acids in the cell-protein. It seems possible that the Gram-positive cell represents an evolution from the Gram-negative form such that the evolved form compensates for the loss of synthetic abilities by this concentration of amino-acids within the internal environment in roughly the right proportions for anabolic purposes. There is little doubt but that Gram-negative cells can assimilate amino-acids—the nutrition of *Eberthella typhosa*, *Shigella* and the *Neisseria* proves that,—but they cannot effect the internal concentration. The substance responsible for the positive Gram reaction, the Gram complex, has been studied by Henry & Stacey (13, 14) in England and by Umbreit & Bartholomew (15) in this country. It appears to be a protein with a prosthetic group consisting of Mg ribonucleotide and appears to reside mainly in the surface layers of the cell. If this substance is intimately bound up in the assimilation-and-concentration process, then we may have an explanation of the effect recently shown by Frieden & Frazier (16) of magnesium in increasing penicillin sensitivity, since reduction of magnesium would promote the growth of mutants of a less-Gram-positive character which were, consequently, less able to effect assimilation and more able to carry out synthesis.

Penicillin is only effective on growing cells. When the Gram-positive cell grows, it must synthesise the Gram-complex and this requires magnesium and the synthesis of ribonucleic acid. This immediately reminds us of the studies of Krampitz & Werkman (3) on

the effect of penicillin on ribonucleic acid metabolism in *Staph aureus*. These workers showed that penicillin will inhibit the breakdown of either endogenous or exogenous ribonucleic acid, high concentrations of penicillin were used (of the order 400-2000 units/ml) but the workers point out that cell concentrations were used which were about 1000 times the concentrations used in growth inhibition tests. This argument may or may not be valid but there is evidence here that penicillin can disorganise ribonucleic acid metabolism. A suggestion which forces itself upon us at this stage is that penicillin interferes with the synthesis of the Gram-complex or otherwise disorganises the nucleotide composition of the cell such that the concentration and assimilation mechanisms break down. Dufrenoy & Pratt (17) have recently shown that when *Staph aureus* is grown in bacteriostatic concentrations of penicillin, then the cells gradually lose their Gram-positiveness and eventually become Gram-negative. If there is an actual disorganisation of the nucleotide complex, it should be possible to detect some alteration in the ribonucleic acid/desoxyribonucleic acid ratio under the influence of penicillin. Table 7 shows results we have obtained in such an attempt. A normal penicillin-sensitive *Staph aureus* was grown in casein-digest-glucose medium and 5 units penicillin/ml added to portions of the culture at the end of the fourth hour of incubation. Organisms were harvested at the time of the penicillin addition and at intervals of 30 and 120 min after the addition. In all cases the ribonucleic-phosphate and desoxyribonucleic-phosphate were estimated by the method of Schmidt & Tannhauser (18). In the normal culture there is a steady drop in both ribo- and desoxyribonucleic acid content with age of culture, much as described by Malmgren & Heden (19). The addition of penicillin has been followed by a significant decrease in the ribonucleic-P, 6% below the control after 30 min and 10% after 120 min, and a marked increase in the desoxyribonucleic-P content amounting to 20% greater than the control after 30 min and 58% after 120 min. The ratio between ribonucleic-P and desoxyribonucleic-P averages 8.7 for the control culture and has fallen to 6.5 for the culture grown in penicillin for 30 min and to 5.55 for the 120 min penicillin-culture. There is definite evidence here of a disorganisation of ribonucleic acid metabolism.

If we calculate the total amount of nucleic acids per ml of culture

we can see that the synthesis of desoxyribonucleic acid is the same in the presence of penicillin as in its absence while the increase in ribonucleic acid is only 10% in the penicillin culture (after 30 min) compared with an increase of 42% in the normal culture. If the suggestion of Brachet (20) is correct, that ribonucleic acid gives rise to desoxyribonucleic acid, then these results could be interpreted as meaning that penicillin has inhibited the formation of ribonucleic acid but not its transformation into desoxyribonucleic acid. Analyses

TABLE 7 (LECTURE III)

Effect of Penicillin on the Nucleotide Content of Staph aureus

Staph aureus grown at 37°C in casein-digest-glucose medium and 5 units penicillin/ml added to growing cultures as indicated below. All quantities expressed as µg P/mg dry weight of cells

	AGE OF CULTURE					GRAM NEGATIVE RESISTANT ORGANISM
	4 hr	4½ hr		6 hr		
Time for which penicillin was present before harvesting	—	—	30 min	—	120 min	—
Total P content	32.6	30.2	31.7	27.6	26.2	15.2
Ribonucleic acid P	20.2	18.8	17.7	15.5	13.9	8.38
Desoxyribonucleic acid P	2.30	2.26	2.71	1.58	2.5	3.06
Ratio $\frac{\text{Ribonucleic-P}}{\text{Desoxyribonucleic-P}}$	8.8	8.3	6.5	9.8	5.55	2.7
Dry weight of cells in culture (mg/ml)	0.121	0.190	0.154			
Ribonucleic-P/ml culture	2.45	3.47	2.72			
Desoxyribonucleic-P/ml culture	0.25	0.43	0.42			

carried out on the highly resistant Gram-negative organism derived from *Staph aureus* 6773 show that this has the very low ratio of 2.7 while its desoxyribonucleic-P content is higher than that of the Gram-positive organism from which it was derived. The action of penicillin on the Gram-positive organism has thus had the effect of altering its nucleic acid composition in the direction of that of the Gram-negative organism.

It seems probable that we have reached the limit of the knowledge

that studies of assimilation and amino-acid synthesis can give us about the action of penicillin. The present indication is that we must turn our attention to the study of nucleic acid synthesis and metabolism with especial reference to that material laid down in the Gram-positive cell and imparting the positive reaction when treated by the Gram technique. Perhaps, in turn, such studies might throw some light on the part played by this material in the assimilation-concentration mechanism evolved by the Gram-positive bacterium. We have to bear in mind, through all this, that the *Neisseria* are Gram-negative but highly penicillin-sensitive. They may possibly represent organisms which have not yet acquired the internal concentration mechanism. For the moment we must content ourselves with reflecting that the cytologist says that the *Neisseria* are "Gram-intermediate" and that they are certainly unable to synthesise certain amino acids, all the *Neisseria* so far studied being nutritionally exacting towards both cystine and glutamic acid at least.

The studies that I have outlined in these lectures on the assimilation of amino-acids by bacteria have enabled us to gain a certain insight into the nitrogen metabolism of Gram-positive bacteria and they have, at the same time, raised many new problems for us to investigate. It is, however, one of the delights of scientific research that the answer to each problem raises new ones and that, as we pass each stage in our enquiry, we observe that evolution of knowledge which is described, in the words of Spencer, as a "change from a relatively indefinite incoherent homogeneity to a relatively definite coherent heterogeneity."

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SULFHYDRYL COMPOUNDS AND THE SICKLING PHENOMENON

A PRELIMINARY REPORT¹

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The methods which are in current use for the routine laboratory diagnosis of sickle cell disease are based on the widely accepted concept that the sickling phenomenon is brought about by the reduction of hemoglobin in the red cell. Exposure of susceptible cells to high concentrations of carbon dioxide is known to cause rapid sickling (1). Exposure to atmospheres with sufficiently low oxygen tension similarly accelerates sickling (2). The production of prolonged stasis by a tourniquet has been shown to enhance the sickling in samples of venous blood (3). Spontaneous sickling occurs when suspensions of cells are sealed beneath a cover slip and allowed to stand for some hours (4) (5). This latter procedure is probably the one most commonly employed in routine diagnostic laboratories, but it has the disadvantage that a conclusive diagnosis is often impossible until twenty four hours after the test has been set up.

In the present study, the effect of a variety of reducing agents on sickle cells was tested with the purpose of devising a dependable means for the rapid diagnosis of sickle cell disease. It was found that sulfhydryl compounds, notably hydrogen sulfide, BAL (2,3-dimercaptopropanol), and cysteine caused complete sickling within less than a half hour. This report is concerned with a description of these observations, together with a presentation of certain related findings which may have bearing on the underlying mechanism of sickling.

¹ This work was supported by a grant from the Life Insurance Medical Research Fund

MATERIALS AND METHODS

Varying concentrations of BAL,² cysteine hydrochloride,³ and glutathione,⁴ as well as other reagents to be mentioned below, were prepared by dissolving the chemicals in physiological saline and adjusting the pH to neutrality with sodium hydroxide. Hydrogen sulfide was prepared as a saturated solution by bubbling the gas through saline. For the test, a small drop of the reagent was mixed on a slide with a drop of oxalated blood which had been diluted approximately 1 to 5 with saline. The mixture was immediately covered with a glass coverslip and, if prolonged observation was desired, sealed at the edges with vaseline.

Approximately 50 specimens of blood were obtained from patients with known sickle cell disease. As controls, 30 specimens from normal individuals and 6 specimens from patients with other varieties of anemia were tested.

RESULTS

In Table I are summarized the results of testing a sample of blood in the presence of several thiol reagents. These results are illustrative of those obtained in every case of sickle cell disease studied. The most rapid sickling was seen in preparations mixed with hydrogen sulfide, in most instances sickling was clearly evident within five minutes after the mixture was placed on the slide, and the majority of the cells were fully sickled within fifteen minutes. In the presence of BAL and cysteine, sickling usually became apparent in fifteen minutes and was complete within a half hour. Glutathione was considerably less effective, producing satisfactory sickling only after two or three hours. The concentrations of thiol compounds necessary to produce sickling are indicated in Table I. For practical purposes, the most suitable concentrations were found to be as follows: hydrogen sulfide—an approximately saturated solution, cysteine—0.5 molar solution, BAL—0.1 molar solution. It is of interest that in general the concentrations of each substance which produced sickling were also those which gave a positive nitroprusside reaction, and a negative nitro-

² Pure BAL supplied through the courtesy of Dr. Harry Eagle.

³ L-Cysteine hydrochloride (Merck and Co., Inc., Rahway, N. J.)

⁴ Glutathione (Schwarz Laboratories, Inc., New York 17, N. Y.)

prusside reaction always indicated an amount insufficient to cause sickling

The thiol reagents did not produce sickling or any other change in contour resembling sickling in the red cells of normal individuals

TABLE I
The Production of Sickling by Sulfhydryl Compounds

REAGENT	CONCENTRATION	TIME OF OBSERVATION (IN MINUTES)					
		5	10	15	30	60	120
H ₂ S (saturated solution)	1 1	++*	+++	++++	++++	++++	++++
	1 2	+	+++	++++	++++	++++	++++
	1 4	—	++	+++	++++	++++	++++
	1 8	—	+	++	+++	++++	++++
	1 16	—	—	—	+	++	++++
BAL	0 10M	+	++	+++	++++	++++	++++
	0 05M	—	+	++	++++	++++	++++
	0 01M	—	—	+	++	++++	++++
Cysteine	0 50M	—	+	+++	++++	++++	++++
	0 10M	—	—	++	+++	++++	++++
	0 01M	—	—	+	+	++	++
Glutathione	0 25M	—	—	—	—	+	++
	0 05M	—	—	—	—	—	—
NaCl		—	—	—	—	—	—

*+, approximately 5% of cells sickled

++, approximately 25% of cells sickled

+++ , approximately 75% of cells sickled

++++, approximately 90-100% of cells sickled

Blood from three adult negroes with the sickling trait, who gave no history suggesting sickle cell disease, showed the same response to thiol compounds as that from patients with the active disease. However, in two other adults with the sickling trait, the cells exhibited a delay of two hours before responding to hydrogen sulfide or BAL. The possible significance of this delayed response will depend upon the accumulation of additional data with blood from sickle-trait individuals. At the present time, an insufficient number of such persons has been studied.

Oxygen exerted a marked inhibitory effect on the sickling produced by sulfhydryl compounds, and was also capable of reversing the phenomenon. Sickling did not occur in preparations left open to the air, or in ordinary hanging drop preparations. When the coverslip overlying a fully sickled preparation was removed, the sickled cells rapidly and completely reverted to the normal discoid shape.

The effect of reagents known to be thiol antagonists was tested with several samples of sickle cell blood. It was found that spontaneous sickling, as well as thiol-induced sickling, was completely inhibited by small amounts of o-chloromercuribenzoate,⁵ iodoacetamide,⁵ iodosobenzoate,⁵ hydroquinone and sodium maleate. The interpretation of the inhibitory effect on spontaneous sickling is not yet clear. It is possible that these substances may block an —SH mechanism within the cell, but it is also possible that the effect is due to some other less specific chemical trauma to the cell. Further investigations of the effect are in progress.

The thiol reagents caused a pronounced darkening in the color of the blood within a few minutes after mixing. This color change was always seen with concentrations of thiol compounds which were sufficient to produce sickling. It was accompanied by the disappearance of the absorption bands of oxyhemoglobin, visualized with a spectroscope, and by the appearance of the typical absorption spectrum of reduced hemoglobin.

The erythrocyte sedimentation rate was tested in three patients with the active disease, comparing oxygenated blood with other samples of blood which, following oxygenation, were treated with BAL or hydrogen sulfide. It was found in each instance that hydrogen sulfide and BAL caused a marked reduction of the sedimentation rate, which was of the same degree as that caused by carbon dioxide (6) (7). It was also noted that rouleaux formation did not occur in the samples treated with the sulfhydryl reagents, although it was prominent in untreated samples.

COMMENT

The possible significance of the foregoing observations in regard to the mechanism of sickling is the subject of an investigation which is

⁵ Obtained through the courtesy of Dr. Leshe Hellerman.

still in progress. The detailed results of this investigation will be reported at a later date.

For practical purposes, the findings provide a new method by which a rapid and reliable diagnosis of sickle cell disease can be made. In our experience, the simplest and most rapidly active agent of the group tested is the saturated solution of hydrogen sulfide. When a drop of this reagent is mixed on a slide with a drop of blood, sickling of a majority of the cells is evident within fifteen minutes and is not infrequently complete in as short a time as five minutes. It has been found that solutions of hydrogen sulfide maintain their activity for five days if kept in the refrigerator in tightly stoppered containers. BAL and cysteine, on the other hand, are highly unstable and must be prepared in fresh solutions each day and kept cold at all times. The activity of any of the reagents can be verified in rough fashion by means of the nitroprusside test.

SUMMARY

Hydrogen sulfide, BAL and cysteine produce rapid and complete sickling when mixed with the blood of a patient with sickle cell disease. Glutathione causes sickling after a longer interval. Sickling produced by sulfhydryl compounds is rapidly reversed by exposure of the sickled cells to air. The sedimentation rate of oxygenated blood from patients with sickle cell disease is markedly diminished by the addition of hydrogen sulfide or BAL. Spontaneous sickling, as well as sickling by thiol reagents, is inhibited by certain agents known to be thiol antagonists.

It is suggested that sulfhydryl compounds may be of value in the laboratory diagnosis of sickle cell disease because of the rapidity with which reliable and reproducible results can be obtained.

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BOOK REVIEWS

(These reviews represent the individual opinions of the reviewers and not necessarily those of the members of the Editorial Board of this journal)

Brain and Intelligence By WARD C HALSTEAD 206 pp \$6 00 *University of Chicago Press, Chicago, Ill*, 1947

Although *Brain and Intelligence* undoubtedly will not be one of Chancellor Hutchins' 100 books, it is nevertheless a much needed argument for objectivity in the field of psychological and psychiatric testing. The author makes his central theme "the frontal lobes of the brain, long regarded as silent areas, but in reality the organs of civilization—the basis of man's despair and of his hope for the future." Halstead has utilized his opportunities in the neurosurgical clinic at the University of Chicago, where he saw patients of Walker, Bucy, Bailey and others before and after various brain operations. Among these he lists cerebral lobectomies, military head injuries, closed head injuries, pre- and post-lobotomies—a total of 207 patients. He applied a number of objective tests, 27 "behavioral indicators", described in an appendix of the book. The volume contains an excellent group of diagrams of the brain lesions of the cerebral lobectomies.

The book begins with a review of some of the neurological, psychiatric, and psychological work leading up to the author's concepts and his argument for "biological intelligence." Previous studies are grouped under psychometric, clinical, and neurological intelligence. With considerable hauteur the author makes short shrift of nearly all past work with the exception of that of Lashley and one or two others. "Professor Halstead has combined new instruments and quantitative methods with the techniques of factor analysis [Thurstone and Holzinger] to isolate the basic factors of higher mental processes," says the jacket review. The results of the author's tests are described in chapters entitled the A Factor, the Power Factor P, the Directional Factor D. Then comes a kind of philosophy of the results under the title "Nuclear Structure of the Ego." Part II deals with "Localization of Function in the Brain."

While some readers will doubtless welcome the relegation of the data to a higher level of discussion and its embellishment by the use of such terms as the Field Factor A, the Power Factor P, the Directional Factor D, and the Nuclear Structure of the Ego, the present reviewer is somewhat bewildered by these highfalutin adornments. The state of confusion in our present thinking and testing which Halstead's data might go far toward dispelling is partly reincarnated by his own special brand of a higher psychological philosophy. The tests are dissected into 26 "quantitative indicators" (p 171), 17 of which bear the name of the author, thus 15 is "Halstead Time-Sense Test (Vision)", 16 is "Halstead Time-Sense Test (Memory)", 17 "Halstead Dynamic Visual Field Test (Central Form)". Since many of these tests have been previously used under other names and are not

sufficiently modified here to warrant a rebirth under a new nomenclature, one is irritated to see work as good as Dr Halstead's promises to be thus marred

Like Little Jack Horner eating his Christmas pie, Halstead has stuck in his thumb and pulled out a plum in his emphasis on the necessity for objective testing, and perhaps he can be excused for the "What a fine boy am I!" This Jack Horner attitude pervading the book, howsoever annoying, should not blind us to the need for such an approach, and the author is to be commended for his enthusiastic argument for objectivity and a demonstration of how objective tests may reveal important data

W H G

Fundamentals of Psychiatry 4th edition By EDWARD A STRECKER 325 pp
\$4 00 J B Lippincott Co, Philadelphia, Pennsylvania, 1947

The latest revised edition of this elementary text is likely to enjoy considerable popularity, as have other editions in the past. Among new chapters, the one on military psychiatry contains an excellent summary of psychiatric knowledge gained during World War II, including a critical evaluation of the subject. The section, "Psychosomatic Medicine and Psychiatry", is practically identical with a corresponding chapter in Strecker, Ebaugh and Ewalt, "Practical Clinical Psychiatry," 5th edition, and gives little clarification of the matter. Although the book is dedicated to the practitioner of medicine, he will find it difficult at times to make ready use of its contents, particularly as regards the treatment of mental disorders. The author's inclusion of a classification of psychiatric illness based on "tension" constitutes an interesting attempt to popularize this terminology. Because of Dr Strecker's appealing style and his awareness of his audience's needs, the book makes easy and good reading and should stimulate the student's interest to tackle the more complex aspects of psychiatry.

E A

Headache and Other Head Pain By HAROLD G WOLFF 642 pp 152 figures
\$12 00 Oxford University Press, 114 Fifth Avenue, New York 11, New York,
1948

In this volume Dr Wolff records the results of 15 years' study of the natural history of headache, by far the commonest and one of the most distressing of human discomforts.

In an introductory chapter the author grapples with the old, unanswered question of the nature of pain and, after 50 pages, 23 figures, and 125 references, returns to the old view that, to the victim, the quale or feeling state is the most important aspect. He then proceeds to a descriptive analysis of the pain—sensitive structures within the cranial cavity, much of this together with data on the localization and nature of the resultant pain emerged from systematic stimulation of these structures during craniotomy.

Confirming and extending the observations of Cushing and others he has demon-

strated that, with the exception of parts of the dura at the base of the brain, sensitivity to pain is limited to venous sinuses and their tributaries, dural arteries and major arteries at the base. Upon this the author derives six basic mechanisms of headache arising within the cranial cavity: (1) Traction on veins passing from brain surface to venous sinuses and displacement of great venous sinuses; (2) Traction on middle meningeal arteries; (3) Traction on large arteries at base of the brain or their main branches; (4) Distention and dilatation of intracranial arteries; (5) Inflammation in or about pain-sensitive structures of the head; and (6) Direct pressure by tumors or adjacent tissue on cranial or cervical nerves, containing many pain-afferent fibers. These he then applies to the explanation of various types of headache.

Most exhaustive (140 pages) is the section on the migraine syndrome which, it is concluded, results from distention of cranial arteries. At the same time the somewhat imponderable nature of the syndrome is implied in its description as "a pattern of dysfunction integrated within the nervous system and manifested as mood changes and widespread bodily disturbances of a non-painful and painful nature."

The book sifts critically the published observations and conclusions regarding head pain and adds an enormous amount of pertinent data obtained by relatively simple experimental approaches to simple questions. The results serve by inference to identify the source of many types of headache and to point the way to treatment.

Illustrations are plentiful and lucid. Most welcome are the brief summaries at the ends of the chapters and a final chapter on clinical differentiation.

E C A

Operative Gynecology 6th edition By HARRY S. CROSSEN AND ROBERT J. CROSSEN. Illus. 999 pp. \$15.00. C. V. Mosby Company, St. Louis, Missouri, 1948.

This is the newest edition of a book which, until recently, was one of the very few authoritative books on this particular division of surgery. It is thoroughly illustrated, and many of the additional illustrations have been reprinted from articles and other books. The color drawings contrast sharply with the excellent colored photographs. There has been considerable reorganization and rearrangement of the chapters in line with the recent advances in gynecology. The subject of uterine cancer and the discussions of x-ray and radium treatment are particularly clear and complete. The authors have given long and critical consideration to carcinoma of the vulva, in line with Taussig's studies and recommendations. The chapters on retrodisplacement of the uterus, prolapse of the uterus, and genital fistulae are particularly thorough. The addition of a short historical sketch and the references to the recent literature on each subject are welcome and useful to both the specialist and the general surgeon. Gynecologists will heartily endorse the authors' constant plea for removal of the involuting or involuted uterus and ovaries.

when an operation is necessary. The cancer potential of these organs is stressed and certainly there can be little excuse for removing only one ovary in a post-menopausal patient or for leaving the uterus in a forty year old patient who has been rendered sterile or castrated by the operation. Dr H S Brookes, Jr has again written the chapters on "The Intestinal Tract" and "Anesthesia".

A few minor criticisms can be made. The sulfa drugs and the new antibiotics are given scant attention and little notice is taken of the ways in which such therapy has altered surgical treatment. Sulfasuxidine was not recommended preoperatively or postoperatively in the treatment of rectovaginal fistulae. The cervical biopsy clamp and procedure are declared out of date, the substitute offered (sharp conization) is often necessary, but cannot practicably replace this simple office instrument and its usefulness. In view of the fact that tuberculosis of the uterus is almost always secondary to tuberculosis of the tubes, the advice on cervical amputation for tuberculosis of the cervix and hysterectomy for tuberculosis of the uterus is misleading and confusing. Sodium pentothal has been the greatest recent advance in gynecological anesthesia, yet it is hurriedly and incompletely discussed.

The authors have inserted much new material and the book is a very useful one for gynecological surgeons and surgeons who occasionally do gynecology.

R B S

Psychobiology and Psychiatry 2nd edition By WENDELL MUNCIE Illus 620 pages \$9 00 *The C V Mosby Company, St Louis, 1948*

In this revised edition the general plan and orientation of the first edition have been retained. The long section, 184 pages, of historical parallels along national lines has been omitted. Thus shortened, and printed on thinner paper, the book is physically more attractive and easier to handle.

The new section on treatment has been enlarged and includes a discussion of group therapy. The portion dealing with classification presents the revised Army nomenclature, keyed to the concept of reaction patterns. There is a thirty-page section on "Somatization Reactions or Psychosomatic Reactions".

The distinguishing feature of Dr Muncie's book is the systematic presentation of Meyerian psychobiology and the ergasiologic formulation of clinical syndromes. One of the major difficulties in preparing a modern textbook of psychiatry is the problem of correlating and integrating psychodynamic considerations with the descriptive delineation of reaction types. For many students this difficulty is somewhat increased in this textbook by the plan of organization in which the psychodynamics are somewhat segregated in the first part, dealing with normal behavior, and the second part is left rather starkly descriptive. For the more advanced student, prepared to utilize "genetic-dynamic" concepts in an integrated mastery of the materials presented, Dr Muncie's book offers a treasury of case material, with many wise and penetrating comments, and a generous selection of pertinent references to American and European authors.

J C W

Unipolar Lead Electrocardiography By EMANUEL GOLDBERGER Illus 182 pp
\$4 00 Lea & Febiger, Philadelphia, Pennsylvania, 1947

Unipolar lead electrocardiography offers certain advantages over conventional methods. The conventional electrocardiographic leads are bipolar, recording the difference in potential between the two electrodes. Wilson in 1934 devised a unipolar lead so that the indifferent electrode has a theoretical potential of zero. Such a lead, therefore, represents primarily the potential at the exploring electrode, enabling one to record the potential at any one point rather than between two points on the body.

An increasing number of original articles attest to the growing knowledge in this comparatively new field. Goldberger's is the first text-book to be published on this subject. Much of this book is devoted to a detailed description of the electrocardiographic illustrations. Flagrant errors are noted when one attempts to check the descriptions with the actual electrocardiograms. Normal patterns are referred to as abnormal. Abnormal records are described as normal. The text description contradicts the labels under the illustrations. P and R waves described as of high voltage are actually within the normal limits quoted by the author. The author's chief difficulty seems to lie with the T waves, and he frequently describes as "downward" T waves which are actually upright, isoelectric or diphasic.

There are many vague, loose and incorrect statements. On page 61 the statement appears, "When the heart is vertical, $P_{1\text{ arm}}$ is frequently downward, but exceptions to this statement are common." However, in illustrations of 10 normal vertical hearts, $P_{1\text{ arm}}$ is downward in only 2, upright in 4, isoelectric in 4. In discussing left ventricular strain (page 94) one finds, "in this case, therefore, the presence of ventricular hypertrophy was indirectly shown by the electrocardiographic signs of auricular hypertrophy," (large P waves). Again, on page 100, "This tracing, therefore, only indirectly shows the presence of right ventricular hypertrophy due to the presence of the electrocardiographic patterns of auricular hypertrophy." The author twice states that when a myocardial infarct heals, the RS-T segment and T waves become normal. Not all cardiographers will accept this or many other statements in this text.

The book is poorly organized. The only illustration and description of a unipolar electrocardiogram in the section on congenital cardiovascular disease is that of dextrocardia. Insufficient evidence is offered for many of the statements in this book.

This volume fails to cover the subject thoroughly or accurately. It is, therefore, not recommended. There is still a need for a good text-book on unipolar electrocardiography.

DIFFERENTIAL DIAGNOSIS OF TUMORS AT THE CEREBELLOPONTILE RECESS

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The diagnosis of a lesion occupying the cerebellopontile recess is relatively easy. The clinical differentiation of these lesions represents, however, a difficult problem which frequently is not solved until a microscopic examination of the pathological specimen is undertaken. We have confronted this diagnostic dilemma during our previous study on the neurinomas (8) and decided, therefore, to analyze the different tumors occurring in this location and to point out, whenever possible, their main differential diagnostic features.

As seen in the accompanying table a total of two hundred and five tumors of the cerebellopontile recess were verified at operation at the Johns Hopkins Hospital from 1926 to 1945. Most of these operations were performed by Dr. Walter E. Dandy. The most common type was the neurinoma which comprised 78 per cent of the series. The remaining 22 per cent of neoplasms will be discussed in detail in this presentation as we have analyzed the neurinoma group elsewhere (8). Abscesses have been included in the series because clinically they may behave like any other tumor at the angle. Aneurysms and other vascular abnormalities purposely have not been considered as we wish to limit ourselves at this time to lesions which may be attacked surgically with certain degrees of success. There are many other lesions at the recess not included in this paper, such as the infectious granulomas, the osteomas, and the so-called chronic cisternal arachnoiditis. None of these were found in the hospital records during the period under consideration. The classification of Evans and Courville (6) on the subject is quite complete and the reader is therefore referred to their paper.

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NEURINOMAS

Only the outstanding characteristics of the neurinomas shall be discussed because, as we stated before, they have been analyzed in detail in a previous communication (8)

a Unilateral acoustic neurinomas without peripheral neurofibromatosis one hundred and forty five such tumors were found in this

TABLE 1
Cerebellopontile tumors The Johns Hopkins Hospital 1926-1945

TYPE OF TUMOR	NO CASES	%
Neurinomas	160	78 0
a Unilateral acoustic	145	
b Bilateral acoustic	6	
c Unilateral acoustic with peripheral neurofibromatosis	3	
d Neurinomas Vth N	3	
e Neurinomas Xth N	2	
f Neurinomas XIth N	1	
Cholesteatomas	13	6 3
Meningiomas	13	6 3
Gliomas	12	5 9
a Astrocytoma	5	
b Astroblastoma	1	
c Medulloblastoma	3	
d Ependymoma	2	
e Glioblastoma multiforme	1	
Abscesses	4	1 9
Miscellaneous tumors	3	1 6
a Sarcoma of meninges	1	
b Carotid body tumor	1	
c Malignant melanoma	1	
Total	205	100 0

series, 62 8 per cent of which occurred in females and 37 2 per cent in males. The average age at the onset of symptoms was 39 6 years, the youngest 12 and the oldest 69. The average duration of symptoms was 4 5 years, the shortest 8 weeks, and the longest 20 years. The chronological order of symptoms postulated by Cushing (2) was present in only 31 per cent of the cases. In 30 per cent the Vth N was affected shortly after the onset of auditory and labyrinthine disturbances. Incoordination and other manifestations of involvement

of the cerebellar pathways followed symptoms referable to the VIIIth N in 20.7 per cent. Headaches, occipital pains or suboccipital discomforts preceded even the auditory manifestations in 12.4 per cent. In 6 per cent no definite pattern was followed.

In the greatest number of instances there was subjective and objective evidence of involvement of the VIIIth and Vth nerves as well as the cerebellar pathways. The VIIIth N was involved in every case either by affection of both components or by solitary involvement of either the cochlear or vestibular branches. In certain instances only the VIIIth N was affected, the patients presenting on admission Meniere's syndrome, which although uncommon with tumors at the angle does occur. Tic douloureux was the only complaint in many instances, but in this series was always accompanied by some loss of hearing, with the exception of one case. This hearing deficit may be easily overlooked because of the frequency of hearing loss at this age. Audiometer and vestibular tests are therefore in order in every patient with tic douloureux. X-ray evidence of destruction of the porus acusticus was present in 50 per cent of the cases and when absent was not therefore absolute proof against a positive diagnosis. Although papilledema has been mentioned by others (9) as a very common finding in acoustic tumors, it was found in only 66.5 per cent of this group.

b Unilateral acoustic neurinomas and peripheral neurofibromatosis these have been considered separately from the preceding group as they differ in some respects. Only three such cases were found. The average age at the onset of intracranial disturbances was far below the average age of patients with solitary neurinomas, e.g., 14.3 years, the youngest 9 and the oldest 20. The duration of symptoms was 10 years, twice as long as in the preceding group.

There was no specific chronological order of symptoms but the auditory, trigeminal and cerebellar manifestations were predominant. The first two were constantly present objectively. Only one patient showed involvement of the spinal cord.

c Bilateral Acoustic Neurinomas six such tumors were encountered. Males and females were equally affected. In two of these patients, both sisters, there was a history of bilateral acoustic neurinomas on the maternal side of the family in approximately thirty-

five members since the eighteenth century Their relationship with peripheral von Recklinghausen's disease seems more obvious than in the unilateral neurinomas Three cases showed evidence of peripheral involvement elsewhere in the body

The average age at the onset of symptoms was 17.8 years, the youngest 9 and the oldest 29 These figures approach those found in unilateral acoustic tumors with evidence of peripheral neurofibromatosis The duration of symptoms, however, was much shorter, e.g., 5 years, approaching the duration of symptoms in the unilateral neurinomas without von Recklinghausen's disease

The symptomatology was typically characterized by bilateral diminution of hearing which was corroborated objectively in all Bilateral involvement of one or more cranial nerves and cerebellar pathways was invariably present X-ray of the skull showed unilateral or bilateral erosion of the foramen acusticus

d Neurinomas of the Vth Nerve three cases comprise this group There was no evidence of peripheral neurofibromatosis The age of onset was much later than in the solitary acoustic neurinomas, e.g., 47 years, and the average duration was exactly the same as in the acoustic neurinomas with evidence of peripheral neurofibromatosis

Upon admission to the hospital these cases were clinically indistinguishable from the unilateral acoustic neurinomas The only important differential diagnostic point was the fact that trigeminal symptoms preceded auditory and labyrinthine disturbances by an average of 4.5 years In two of these patients the initial and predominant complaint was that of tic douloureux, and this again stresses (4, 5) the importance of the relationship of tumors at the cerebello-pontile recess to trigeminal neuralgia

e Neurinomas of the Xth Nerve two such cases were found in this series The age at the onset of symptoms was 29 and 53 years respectively and the duration of symptoms 1 and 7 years The predominant and initial symptom was that of hoarseness which in one case was the only finding Paresis of one vocal cord was corroborated on admission Evidence of involvement of other cranial nerves may or may not be present If hoarseness is associated with unilateral paralysis of the vocal cords a tumor of the Xth N is a likely possibility

f Neurinomas of the XIth Nerve only one case was encountered in

this group The age at the appearance of symptoms was 63 5 years with a duration of $4\frac{1}{2}$ years There was evidence of peripheral neurofibromatosis about 5 years previous before the onset of intracranial involvement The initial symptom was gradual stiffness and atrophy of the left arm and shoulder girdle, followed 3 years later by suboccipital discomfort, dysphagia, hoarseness and incontinence On admission the patient showed involvement of the IXth, Xth, XIth and XIIth cranial nerves and ipsilateral cerebellar signs There was no impairment of hearing The presence of a neurinoma originating from the XIth N was disclosed at operation

CHOLESTEATOMAS OR PEARLY BODY TUMORS

Thirteen such cases (6 3 per cent) were present in this series, exactly the same number as the meningiomas which will be discussed later Females were affected more frequently than males in a ratio of 10 3 In one case the symptomatology started with trauma to the head on the affected side with loss of consciousness A history of acute otitis media on the same side as the lesion was elicited in another In the latter case there were no symptoms referable to the ear and no obvious abnormalities could be elicited upon examining the VIIIth N The left side was affected almost invariably, only two cases occurring on the right

The average age of the patients on admission was 40 6 years, the oldest 60 and the youngest 13 The actual age at the onset was 33 3 years, the oldest 41 and the youngest 11 The average duration of symptoms was 7 3 years, the longest 23 years and the shortest 6 weeks

Symptomatology In ten cases the only symptom on admission was that of tic douloureux, one more case having been added since our previous communication (7) The chief complaint in these cases was that of paroxysmal, lancinating, intermittent pain occurring in one side of the face only, affecting the distribution of the three branches of the Vth N in seven, the second and third branches in two, and the first and second in one The pain was more frequent and more severe than in the usual cases with "idiopathic" tic douloureux and less apt to remit None of these ten cases presented complaints referable to the ear The remaining cases had initial symptoms involving the VIIIth N Two complained of tinnitus and gradual diminution of

hearing on the affected side, and one complained of hearing deficit unaccompanied by tinnitus. Two of these cases had severe rotary vertigo coming on in periodic attacks with nausea and vomiting. A diagnosis of Meniere's disease was made and the tumors were found incidentally upon sectioning the VIIIth N through a unilateral cerebellar approach. Cerebellar manifestations were present in three cases. In two they followed the auditory disturbances, and in one the trigeminal complaints. Headaches were similarly quite rare and never represented an initial or predominant symptom. They were described as suboccipital in two and bifrontal in one. Subjective involvement of the VIIth N was even less frequent and was elicited in two cases. In one there was just a peripheral type of facial weakness, and in the other clonic contractions of the facial muscles on the affected side. Dysphagia was present in one case, occurring rather late in the course of the illness, in addition to auditory disturbances.

Signs The state of consciousness was good in every case. Vision was well preserved except in one case with predominant auditory disturbances in which the patient was completely blind on admission and showed on fundoscopic examination secondary optic atrophy. The fundi and peripheral fields were completely normal in the remaining cases. No abnormalities of the IIIrd, IVth and VIth cranial nerves could be elicited in this group. Objectively disturbances of the Vth N were found in four cases. These were characterized by diminished or absent corneal reflexes, hypalgesia and hypaesthesia in one or all of the three branches. Two of these occurred in patients complaining of tic douloureux and the other two in patients who had predominant auditory disturbances. Facial paralysis was infrequent and could be corroborated in only two cases, one of which had only trigeminal symptoms and in the other the auditory manifestations were outstanding. Loss of taste in the anterior two-thirds of the tongue on the affected side was elicited in one patient complaining of tic douloureux with no evidence of facial paralysis. Hearing was intact in all the cases complaining of tic douloureux, and the caloric test was similarly normal in all except one. In three patients with subjective involvement of the VIIIth N the audiogram showed complete deafness in two and partial in one, the hearing deficit being neurogenic. Vestibular tests on the other hand were performed in

only one patient who showed a normal response. The rest of the cranial nerves were intact with the exception of one case with a hearing deficit who showed, in addition, ipsilateral palatal paralysis. Unilateral ataxia and horizontal nystagmus were present in the three cases with predominant VIIIth N disturbances. Horizontal nystagmus alone was found in only one case with tic douloureux. Bilateral pyramidal signs were extremely rare, appearing in one of the cases with auditory disturbances. There were no sensory changes.

X-rays Two patients with tic douloureux were suspected of having a tumor and X-rays of the skull were taken accordingly. Both of these were reported normal. Base films were taken in all patients with auditory disturbances. In one there was definite evidence of erosion of the internal acoustic meatus while the others were entirely negative.

Comments In this group, as in the acoustic neurinomas, females were vastly more affected than males. The left side was almost invariably involved. The duration of symptoms was more prolonged than in the unilateral neurinomas of the VIIIth N without peripheral neurofibromatosis. The most common symptom was that of lancinating pain in one side of the face, intermittent and paroxysmal in character, usually involving the distribution of the three branches of the trigeminal nerve. This pain was less apt to remit spontaneously than in the so-called "idiopathic" tic douloureux, and the intensity and frequency of the attacks were quite severe. Involvement of other cranial nerves was infrequent in spite of the relatively large size of these tumors at the recess. Auditory and labyrinthine manifestations were rare and when present tended to take the form of Meniere's disease. Subjective involvement of other cranial nerves or of the cerebellar pathways was practically absent. Objectively one was surprised at the paucity of signs. Papilledema was extremely rare. Only four cases showed objective evidence of affection of the Vth N. Clonic contractions of one side of the face and unilateral facial weakness occurred in isolated cases. Diminution of hearing was also quite rare. Cerebellar signs were very uncommon and pyramidal signs were almost nil. In most of these patients the true nature of the condition was not suspected as the diagnosis of either tic douloureux or Meniere's disease was made from the history and physical findings. These tumors apparently produced little or no erosion of the porus acusticus as evi-

denced either by x-ray or at operation. When auditory or labyrinthine disturbances were present as the initial symptoms it was somewhat difficult to differentiate them clinically from the acoustic neurinomas. The essential differential points are the younger age of onset, the longer duration of symptoms, the absence of headaches and negative x-ray findings. The diagnosis of these lesions is extremely important since they are benign and easily extirpated. Not a single mortality was recorded in this group.

MENINGIOMAS

Thirteen cases (6.3 per cent) comprise this group. As noted before, they occurred with the same frequency as the pearly body tumors. Likewise, females were affected more often than males in the proportion of 10.3. No history of infection was elicited, but there was history of trauma to the head with loss of consciousness in one case. In contradistinction to the cholesteatomas the right side was affected almost invariably, only three cases occurring on the left.

The average age of the patients on admission was 50.3 years, the youngest 21 and the oldest 71. The average age of the appearance of symptoms was 46.3 years, the oldest 69 and the youngest 19. The average duration of symptoms was four years.

Symptomatology. Auditory and labyrinthine disturbances were the initial symptoms in six. Diminution of hearing was the most predominant subjective manifestation of VIIIth N involvement, being present in eight. Tinnitus was less common, occurring in three cases, in two of which it was simultaneous with the hearing loss while it followed vestibular disturbances in one. Dizziness could be elicited in two, in one of which it was associated with hearing loss and in the other represented the only acoustic symptom. Similarly, true rotary vertigo was present in two cases, in one of which there was tinnitus without hearing loss, and in the other both tinnitus and hearing loss. The preoperative diagnosis of Meniere's disease was made in these two cases.

Tingeminal symptoms occurred as frequently as the auditory manifestations and were present in ten cases, representing the initial symptoms in four. The most predominant complaint was that of paroxysms of pain, which was elicited in five. Subjective numbness

of the face was present in the remainder. It is interesting to note that numbness and pain were not present together. The pain was more apt to remit and less frequent than in the pearly body tumors. Tic represented the initial symptom in four cases while numbness followed auditory disturbances in five. In one instance tic douloureux appeared simultaneously with the auditory disturbances.

The next most frequent symptom was that of cerebellar origin. It was present in eight and represented the initial symptom in two. Clumsiness of one arm or unsteadiness in walking was the usual complaint. It occurred simultaneously with trigeminal symptoms in one and followed disturbances of the VIIIth N. in six.

In contradistinction to the pearly body tumors, headaches were a common feature in the meningiomas and occurred in eight cases. It was the initial symptom in one and occurred initially either with the cerebellar or auditory manifestations in three. It is interesting to note that all cases complaining of numbness of the face had headaches, whereas only one case with tic douloureux complained of this symptom. Headaches were mostly suboccipital but in one case they were both frontal and occipital. Two patients complained of pain behind the ear. In half of the cases headaches were increased by coughing, straining or change of position.

Diminution of vision was present in almost 50 per cent of the cases, and one patient was blind on admission. This symptom came as a late manifestation. Involvement of other cranial nerves was rare. Clonic twitchings of the face on the affected side was present in one case who had predominantly auditory disturbances. Dysphagia and dysarthria, although late manifestations, were more frequent and occurred in four cases. There was no subjective evidence of pyramidal tract or sensory manifestations in the body or extremities. Hypothalamic symptoms such as obesity, somnolence, amenorrhea, polydipsia and polyuria were present in one case in whom the diagnosis of pituitary adenoma was suspected, but obviously these were due to pressure from a dilated third ventricle.

Signs Two patients were drowsy on admission. Diminution of vision was corroborated in five, and one patient was completely blind. Bilateral papilledema was present in four. Secondary optic atrophy and profuse retinal hemorrhages in two. There was no significant

variation in the visual fields, and there were no disturbances of the extra-ocular movements

The Vth N was found to be objectively affected in seven cases. Unilateral sensory changes in the face with diminished or absent corneal reflexes were elicited in six, while bilateral involvement was corroborated in one. In two of these cases there was also involvement of the muscles of mastication. Only one patient complaining of *tic douloureux* showed any sensory loss on the face.

Facial paralysis was more common than in any of the preceding groups and was present in seven cases, partial in five and complete in two. In only one could loss of taste on the anterior two-thirds of the tongue without evidence of facial weakness be corroborated.

In nine cases there was either marked diminution or absence of hearing on the affected side. The hearing loss was unilateral in all but in one who showed bilateral diminution of hearing. An absent vestibular response accompanied every case with hearing deficit. One case with normal hearing showed no response to caloric stimulation. Paralysis of the palate was elicited in one.

Objective cerebellar manifestations were present in seven cases, being unilateral in five and bilateral in two. Horizontal nystagmus was predominant in six, while coarse nystagmus was present in only one case. Pyramidal signs were infrequent, appearing in two cases, unilateral and bilateral respectively. Sensory changes were even more uncommon and were present in one on the same side as the lesion.

X-rays were taken in six of these patients and only one showed destruction of the petrous pyramid. In two there was marked enlargement of the sella turcica and the remainder were normal.

At operation all the tumors were totally removed except in two instances in which there was extension of the neoplastic process to the middle and anterior fossae. The operative mortality was quite high, five of them dying immediately after the operation. In fact, the mortality in this group is the highest of the series. This may be explained by the extreme hardness, vascularity and proximity to the transverse sinus of most of these tumors, which make their removal a most arduous and hazardous procedure. Cushing (2) however, considers them one of the most favorable ones at the recess.

Comments In conclusion, we have that females were vastly more

often affected than males. The average age at the onset of symptoms was 46.3 years with a duration of 4 years. The age at the onset was therefore in an older group than in the preceding ones, while the duration of symptoms approached closely the duration in the solitary acoustic neurinomas. The right side was predominantly affected. Auditory and labyrinthine disturbances represented the most common initial manifestation, and at times they were the only symptoms and signs present on admission, making the diagnosis of Meniere's disease almost a certainty. Trigeminal symptoms, although less frequent as initial manifestations, were as frequent in incidence as the auditory complaints. Several cases, in fact, complained mainly of *tic douloureux*, and with this diagnosis they were operated upon. Cerebellar disturbances and headaches were rare as initial symptoms but occurred later with relative frequency. Headaches were more common in this group than in the cholesteatomas and almost as frequent as in the solitary acoustic neurinomas. Objectively we find more or less the same pattern of findings as in the acoustic neurinomas. The most prominent involvement was that of the auditory nerve, although in a few cases there was no affection of either the cochlear or vestibular component in contradistinction to the neurinomas of the VIIIth N. The Vth N was affected next in order of frequency. The facial nerve was more commonly affected than in any of the group. Papilledema and cerebellar signs were more frequent than in the purely body tumors, but as common as in the neurinoma group. Involvement of other cranial nerves, sensory and pyramidal signs were very uncommon. Roentgenologic evidence of erosion at the *porus acusticus* was infrequent.

The differential diagnosis of these tumors from the acoustic neurinomas may be difficult, in many cases, however, we might make an accurate diagnosis if we remember that the meningiomas occur in a higher age group, that the VIIIth N is more commonly affected, and that radiographic erosion of the *porus* is quite rare.

GLIOMAS

Gliomas at the recess are less numerous than the previous tumors in spite of the relative frequency of neoplasms of the glial group arising from the cerebellum and brain stem in childhood and adoles-

cence The locus of origin of these tumors is most likely from either of these two structures, but we could well expect intrinsic gliomas of the acoustic nerve if we bear in mind that the extracannalicular portion of the VIIIth N is covered mostly by glial tissue (1, 10) The occurrence of these intrinsic tumors is extremely rare and a single report was not found in the literature One case occurred in this series arising from the VIIIth N

Twelve gliomas (5.9 per cent) at the cerebellopontile recess were found in this series, and the individual types will be discussed separately as they present interesting characteristics

1 Astrocytomas

Five cases (2.4 per cent) comprise this group, all females Three were present on the right and two on the left No history of trauma or infection could be elicited coincidental to the initial symptoms The average age of the patients on admission was much younger than in any of the preceding groups of miscellaneous tumors, e.g., 29.4 years, the youngest 7 and the oldest 50 The average age at the onset of symptoms was 26.3 years, the youngest $6\frac{1}{2}$ and the oldest 41 The duration of symptoms was 3.1 years, the shortest 2 months and the longest 9 years

Symptomatology The most common subjective disturbances on admission were those referable to the VIIIth N which were present in all cases They represented the initial complaint in four Unilateral diminution of hearing and tinnitus were present together in three and as isolated manifestations in two Vestibular disturbances on the other hand were conspicuously absent Cerebellar manifestations were the next most common and followed in every case the symptoms referable to the VIIIth N This in itself was rather surprising considering that these tumors arise either from the cerebellum or brain stem Trigeminal symptoms were less common and were present in three cases They never represented the initial symptom One patient complained of typical tic douloureux and the other two of subjective numbness of the face on one side Symptoms referable to the VIth N were present in three cases, in one of which they represented the initial manifestation A history of facial weakness was also elicited in three, one of them showing spasmodic contractions of

the facial muscles on the ipsilateral side. The IXth and Xth nerves were subjectively involved in one case. Involvement of the XIth and XIIth nerves was absent. Pyramidal and sensory changes were present contralaterally in one case. Headaches appeared in two cases, generalized in one and bifrontal in the other, and represented the initial symptom in one.

Signs All patients were conscious, alert, and cooperative on admission. The olfactory nerve was not found involved objectively and vision was good in all cases. There was bitemporal hemianopsia for color in one patient in whom X-ray examination showed enlargement of the sella and destruction of its floor, undoubtedly due to pressure from an enlarged third ventricle. Bilateral papilledema was present in two. No abnormalities of either the IIIrd or IVth cranial nerves could be found. Four cases had ipsilateral absent corneal reflexes and sensory changes on the face. Involvement of the VIth N was corroborated in three, ipsilateral in two, and contralateral in one.

The VIIth N was affected in all cases. The facial paralysis was of the peripheral type, partial in four and complete in one. Diminution of hearing was elicited in four patients. No hearing deficit was found in one patient but he complained of tinnitus on admission. It is unfortunate that vestibular tests were performed in only one case with deficient hearing. The caloric response was normal in this case. Involvement of other cranial nerves could not be discovered on examination.

There were bilateral pyramidal signs in one case with contralateral sensory changes. Cerebellar disturbances were constant, being present in all. Unilateral ataxia was uniform, while nystagmus was present in three. The nystagmus was of the horizontal type.

These tumors were fibrillary astrocytomas. Three seemed to arise from the undersurface of the cerebellar lobes, one from the brain stem and another one from the VIIIth N.

Comments Astrocytomas at the angle producing symptoms similar to acoustic neurinomas are rare and occurred in 2.4 per cent of the cases. Females were selectively affected. The average age at the onset was 26.3 years and the duration of symptoms 3.1 years, these figures being definitely lower than the solitary acoustic neurinomas. There was no definite chronologic order of symptoms. Auditory

manifestations represented the initial disturbance in three, diplopia in one and generalized headaches in another. However, the auditory and cerebellar symptoms were the most frequent. Objectively the only constant signs were those referable to the VIIth N and cerebellar pathways, the VIIIth and Vth nerves being affected next in order of frequency. No changes were elicited on X-ray of the porus acusticus. These tumors may be differentiated from the solitary acoustic neuromas by the younger age of the patient, the shorter duration of symptoms, the predominance of VIIth N and cerebellar signs, and by the absence of x-ray changes of the porus acusticus.

2 *Astroblastomas*

Just one case of this kind appeared in our series, comprising 0.5 per cent. For purpose of simplification it is summarized immediately.

Case report F. Q., W. M., 18, Hist. No. U-52914—Admitted on December 27, 1933, with the history that for one year previous to admission he had been complaining of intermittent attacks of projectile vomiting, without nausea, which gradually had become more frequent. Periodic attacks of occipital headaches increased by straining had been present for seven months. Headaches were accompanied by intermittent diplopia. Increasing weakness and clumsiness of the right arm and leg for four months. Marked stiffness of the neck for two weeks. On admission the physical examination was negative. The neurologic examination showed bilateral papilledema with a small hemorrhage in the left disk, horizontal nystagmus, diminished right corneal reflex, diplopia in all fields of vision without definite paresis of E. O. M., normal hearing but absent vestibular response bilaterally, ataxia of right arm and leg, staggering gait and positive Romberg. X-rays of the skull not taken. Preoperative diagnosis: right cerebellar glioma with possible extension to the angle. Operation (Dec. 28) bilateral cerebellar approach. A large tumor found in the right recess. Tumor apparently arose from the cerebellum pressing tightly against brain stem and slightly attached to it. The VIIth and VIIIth cranial nerves were distorted by the mass. Complete removal by careful dissection. Weight $17\frac{1}{2}$ gms. Histological diagnosis: astroblastoma. Postoperative convalescence was good.

When last seen, twelve years after the operation, he was perfectly well except for a residual facial paralysis on the right

Comments The differential diagnosis in this case would not be difficult if we consider the young age of the patient, the prominence of symptoms of increased intracranial pressure from the onset, the short duration of symptoms, the absence of auditory disturbances, and the predominance of signs referable to the Vth and VIth cranial nerves as well as the cerebellar pathways. Marked papilledema was present. The good result in this case proves that it is always worthwhile to attempt a complete removal of gliomas at the angle.

3 *Medulloblastoma*

This group is comprised of three cases (1.5 per cent). One female and two males were affected. In one the symptoms were coincidental with trauma to the head on the affected side. The right side was invariably involved. The average age of the patients on admission was 30.6 years, the youngest 23 and the oldest 38. The average age at the onset was 28.1 years, the youngest 21 and the oldest 33, and the duration of symptoms was 2.5 years, the shortest 5 months and the longest 5 years. Auditory disturbances, peripheral facial weakness and cerebellar manifestations represented the initial symptoms in these three cases respectively. Symptoms referable to the VIIIth N were present in all cases: bilateral tinnitus present in one and unilateral deafness in two. There were no vestibular disturbances. Cerebellar manifestations and subjective involvement of the Vth and VIIth nerves occurred in two cases. Headaches were a late symptom and were present in two. Dysarthria was present in one case. There was no subjective involvement of the pyramidal or sensory tracts.

Signs The patients were conscious and alert on admission. Anosmia was not elicited in any of the cases. Disturbances to the Vth and VIIIth nerves as well as unilateral cerebellar signs were present in all. The corneal reflex was absent in two, and sensory changes on the face were elicited in each case. There were no abnormalities of the extra-ocular movements. Facial paralysis was present in two, being partial and complete in each case respectively. Unilateral nerve deafness was uniformly present. Caloric tests were performed in one case and showed absent vestibular response on the same side.

as the defective hearing. Unilateral ataxia and nystagmus were present in every case. There were no pyramidal or sensory changes in the body or extremities. X-rays of the skull were reported as negative in all the cases.

At operation typical medulloblastomas were found arising from either the brain stem or cerebellum. All patients were discharged in an improved condition, but adequate follow-up studies were carried in only one case who died 5 years after the operation with massive recurrence.

Comments Medulloblastomas are essentially tumors of childhood. It is surprising that the only three cases found in these series occurred at a much later age, e.g., 28, 1 years. On the other hand it is also surprising that in these rapidly growing neoplasms the average duration of symptoms was 2.5 years. These figures correspond closely to the astrocytoma series and are much lower than the unilateral acoustic neurinomas. Although auditory involvement was a constant sign it represented the initial symptom in only one case. Vth N and cerebellar signs were likewise present in all three cases. Papilledema was uniformly absent. No X-ray changes were found. No definite conclusions can be drawn from this small group of cases but these tumors apparently may be differentiated from the acoustic neurinomas by the earlier age of onset, the shorter duration of symptoms, the low incidence or absence of papilledema and the lack of changes at the porus as evidenced by X-ray examination.

4 *Ependymomas*

Two cases (0.9 per cent) occurred in this group, both of them being in males 37 and 48 years of age on admission. The average age at the onset of symptoms was 40 years with a duration of 2.5 years. The right and the left side were respectively affected. Diminution of hearing and numbness of the face were the initial symptomatology in one, while severe generalized headaches represented the first manifestations in the other. Dysphagia, dysarthria and cerebellar disturbances were present in both, occurring as late manifestations. There was history of intermittent contraction of the face in one.

Signs On admission both patients were alert and cooperative with no impairment of vision and no papilledema. Unilateral involvement

of the Vth, VIIth, VIIIth, IXth and Xth cranial nerves was present in both cases, as well as unilateral cerebellar signs. Horizontal nystagmus could only be elicited in one. Vestibular tests were performed on one who showed no response on either side. X-rays of the skull were normal in one while the other showed erosion of the porus acusticus.

At operation typical ependymomas were found at the angle extending from the fourth ventricle into the recess. One patient is living and well at the end of one and a half years. No follow-up studies were carried on in the other.

Comments. The age of these two cases falls approximately in the same group as the unilateral acoustic neurinomas, but the duration of symptoms was much shorter. In one the diagnosis of an acoustic neurinoma could have been easily ruled out as auditory disturbances were a late manifestation. Another differential point from the acoustic neurinomas in both cases was the absence of papilledema in the presence of such marked involvement of the cranial nerves.

5 *Glioblastoma multiforme*

They were extremely rare in this series which is not surprising if we consider that they are primarily tumors of the cerebral hemispheres. Only one case (0.5 per cent) was found.

Case report. J. F. K., W. M., 37, Hist. No. 11874—Admitted Apr. 30, 1927, with the history that for four years he had been suffering from gradual diminution of hearing on the right which became complete in six months. One year after the onset of hearing difficulties he had paralysis of the right side of the face which was complete in a few days and subsequently remained unchanged. For two and a half years there had been intermittent headaches at the vertex accompanied by dizziness whenever he moved his head. Gradual blurring of vision for one year. On admission the physical examination was negative. The neurologic examination revealed bilateral papilledema, horizontal nystagmus, absent right corneal reflex and hypesthesia of right face, complete peripheral VIIth N. paralysis, complete right nerve deafness, absent vestibular response on the right, dysmetria and asynergia in right arm and leg and a positive Romberg. X-rays of the skull not reported. Operation (May 5) bilateral cerebellar ap-

proach Ventricles were large and fluid spurted under considerable pressure A large tumor was found at the right recess invading the spinal canal It was slightly attached to the brain stem from which it was dissected The tumor appeared to be attached to the dura and indented the internal auditory meatus It was completely removed Weight 39 gms Histological diagnosis glioblastoma multiforme Postoperative convalescence was good and the patient was discharged on the 24th postoperative day No follow-up

Comment It is inconceivable that a tumor of this kind should have a history of four years' duration although it might have represented in its incipient stage one of the more benign gliomas which underwent malignant changes later Clinically it was difficult to distinguish from the unilateral acoustic neurinomas except for the earlier age at the onset of symptoms and by the fact that the facial paralysis was complete This case proves how futile at times a clinical differentiation may be

ABSCESES

As mentioned above, abscesses, although inflammatory in nature, may behave like any tumor at the recess and therefore must be considered in the differential diagnosis Four such cases (2 per cent) comprise this group Two of them were metastatic, secondary to a pyogenic focus elsewhere in the body, while the other two were direct sequelae of a middle ear infection Three males and one female were affected The right and left sides were involved in an equal number of cases

In the otogenic abscesses symptoms appeared one week and nine years respectively after the onset of the primary infection In cases where the primary source was remote, e g, osteomyelitis of the femur, the onset of symptoms started 6 and 15 months respectively after the original infection The average duration of symptoms in three was three weeks, while in the other was four years The average age on admission was 17.5 years, the youngest 11 and the oldest 22 The age at the appearance of symptoms was 16.1 years, the youngest 10 and the oldest 21.5 Facial paralysis was the initial symptom in two, unilateral diminution of hearing in one, and generalized headaches in another Cerebellar manifestations were present in two, appearing

late in the course of the disease. There was no subjective involvement of the Vth N in any of the cases. Unilateral diminution of hearing was present in one case and diplopia in another. Otherwise subjective manifestations of cranial nerve involvement were rare.

Signs Only one case was drowsy on admission, the others were alert and cooperative. Varying elevation of temperature was present in three with moderate leucocytosis. Bilateral papilledema with hemorrhages and exudates was found in three and bilateral IIIrd N palsy in one. Unilateral absence of the corneal reflex was elicited in one and bilaterally in another. No sensory changes on the face could be demonstrated. Paralysis of the VIth N occurred in one case on the same side as the lesion. Peripheral facial paralysis was the most constant sign. It was found in all, complete in two and incomplete in the remainder. Hearing was absent in two with total loss of vestibular response. The left vocal cord was affected in one. Pyramidal signs were present in two, always contralateral to the lesion. Cerebellar signs were as common as the VIIth N involvement occurring in all. The ataxia was unilateral and on the same side as the lesion. Nystagmus appeared in two cases.

X-rays of the skull were uniformly negative.

At operation the unilateral cerebellar approach was used in two and the bilateral in the others. Only the patients in whom the bilateral approach was used survived and are still living one and four and a half years respectively after the operation. In two the offending organism was *Staphylococcus aureus*, in one *Staphylococcus albus hemolyticus*, and no cultures were reported in the other.

Comments Four cases with abscesses at the cerebellopontile recess have been analyzed. In one case the diagnosis of acoustic neurinomas was made due to the long duration of symptoms and to the predominance of the auditory disturbances, although another lesion should have been suspected if the early age of onset had been considered. In the other three the diagnosis of an abscess at the angle was suspected due to the early age of the patients, the short duration of symptoms, the febrile condition with leucocytosis and the unilateral neurologic signs. In all cases a primary pyogenic focus was found either in the middle ear or was an osteomyelitic process in the long bones. Therefore, a young patient with a focus of infection in the

body presenting unilateral cranial nerves and cerebellar involvement should be regarded potentially as harboring an abscess at the cerebellopontile recess

How infection finds its way into the angle has been explained by others (6) In the otogenic abscesses two avenues of spread have been described 1 osteomyelitis of the petrous tip, and 2 by local extension through the internal auditory canal Thus a Gradenigo's syndrome may precede the symptoms of a lesion at the recess

When the abscess is secondary to a remote infection, it must be metastatic, the metastases taking place in all probability to a cerebellar lobe from which it invades the angle or ruptures into it producing a localized collection of pus surrounded by dense fibrous adhesions

MISCELLANEOUS TUMORS

This group is comprised of three cases a sarcoma of the meninges, a carotid body tumor, and a malignant melanoma which are summarized below

1 Sarcoma of the meninges

Case report A R , W M , 11, Hist No 356553—Admitted July 6, 1945, with the history that nine months before admission he had a sudden paralysis of the right side of the face which was diagnosed as a Bell's palsy One and one-half months later almost constant attacks of rotary vertigo, bifrontal headaches, nausea and vomiting and some staggering on walking appeared The diagnosis of right otitis media and mastoiditis was made elsewhere and a simple mastoidectomy on the right performed, followed by a simple mastoidectomy on the left one month afterward Following these operations the vertigo disappeared, but the headaches and vomiting persisted with subsequent loss in weight One month before admission he developed a bilateral convergent squint and the staggering became worse and more pronounced to the right Vomiting had been almost continuous for a week On admission the physical examination showed a chronically ill boy with a normal temperature and white cell count There was no evidence of chronic ear infection A marked laceration of the right cornea was found The neurologic examination showed moderate engorgement of the retinal veins, bilateral VIth N paralysis, complete

on the right and partial on the left, absent right corneal reflex, analgesia and anaesthesia on the whole right side of the face, marked weakness of the right masseters and pterygoids, complete right peripheral facial paralysis with loss of taste on the anterior two-thirds of the tongue, neurogenic type of deafness on the right, paralysis of the right palate, and marked ataxia on the right. X-rays of the skull showed a destructive process involving the right petrous ridge, extending along the middle fossa, obliterating the foramina in the base and extending as far forward as the posterior border of the anterior fossa. First operation (July 7) right cerebellar approach. Resection of the cerebellar cap. A large, reddish, extremely hard tumor was found at the recess which was considered inoperable and no attempt was made to remove it. Postoperatively patient's condition was worse with increasing drowsiness and three days later it was decided to make an attempt to remove the neoplasm. Second operation (July 10). The wound was reopened. The tumor had made a tremendous erosion at the porus and could not be shelled from the base even after liberating the margins. A large part of the tumor was cut away with the knife and scissors. Postoperatively the child never regained consciousness and died twenty-four hours after. Permission for necropsy was not granted. Histological diagnosis of the tumor sarcoma of the meninges.

Comments It was obvious from the history of the patient that this did not represent an acoustic neurinoma. His age, the early appearance of symptoms of increased intracranial pressure, the complete facial paralysis and the rapid progression of symptoms made the diagnosis of a neurinoma quite improbable. The possibility of an abscess at the recess was considered but was discarded in the presence of such marked erosion of the base of the skull which made the diagnosis of a highly malignant lesion, possibly an osteogenic sarcoma, the most likely.

2 Carotid body tumor (*perithelioma of the carotid gland*)

Although there are several instances in the literature of tumors arising from the carotid gland invading and eroding the skull, a single case was not encountered where this kind of neoplasm was found within the cranium without demonstrable signs of its presence

in the neck The following case is an example of such an occurrence which is reported in detail because of its rareness

Case report W B , W M , 38, Hist No U-7229—Admitted Aug 30, 1926, with the history that twelve years previously he had developed sudden hoarseness with slight dysphagia which had since remained unchanged Two years later, following an attack of influenza, he became deaf on the right The tympanic membrane was punctured at that time, and following this an intermittent, chronic discharge had been present He had had for eight years periodic attacks of vertigo (? rotary) accompanied at times by staggering to the right These attacks occurred in the beginning about twice a month, but for one year previous to admission they occurred from two to three times a week, followed at times by profound unconsciousness lasting for one hour Persistent diplopia for eight months Severe suboccipital headaches which had been almost constant, accompanied by marked exacerbations of vertigo, at times so severe as to keep him awake most of the night, had been present for four weeks Two days before admission he became increasingly drowsy On admission the physical examination was essentially negative No masses were palpable in the neck and there were no visible pulsations The neurologic examination showed bilateral papilledema of 1 D , more pronounced on the right, complete right VIth N paralysis, complete neurogenic deafness on the right with absence of vestibular response on the same side, paralysis of the right palate and vocal cord, marked atrophy and paralysis of right sternomastoid and trapezius, atrophy of right side of the tongue and slight dysmetria and asynergia of the right arm X-rays of the skull showed an enlarged sella turcica but no data was given as to views of the base Preoperative diagnosis cerebello-pontile angle tumor Operation (Sept 4) bilateral cerebellar approach A big tumor found in the right recess There was furious bleeding from the tentorial veins on retracting the cerebellum, so much in fact that a quick enucleation of the tumor with the finger was attempted Enucleation was difficult The tumor had eroded the base of the skull and apparently invaded the deep muscles of the neck and extended into the lateral sinus Uncontrollable, exsanguinating hemorrhage occurred and the patient died on the operating table Autopsy was obtained but unfortunately was limited to the head

Autopsy findings There was a ragged hole about 2-3 cm in diameter in the base on the skull at about the position of the jugular foramen on the right and mushy-looking tissue could be seen projecting from it. Some of the tissue in the neck was removed through the hole in the skull, but it looked like normal muscle. Between cerebellum and medulla on the right there was a gouged-out cavity from which a tumor had been removed. On section the aqueduct was compressed and distorted but not occluded. No evidence of tumor was seen invading the brain stem or cerebellum. Microscopic examination "Tumor has a framework which divides it up into rather small areas which are somewhat rounded and apparently surrounded by a continuous cleft. There are channels like blood vessels running through such areas. They have rather thick walls and not very conspicuous endothelium. The remainder of such areas is made up of strands of small masses of quite large cells with vesicular nuclei which do not stain very deeply, and with a finely granular cytoplasm suggesting the presence of fat. In sections of tissue from the neck the tumor is found in a large vein. No tumor tissue can be seen in the lymph glands." **Diagnosis** carotid body tumor

Comment This case might have been diagnosed as a neurinoma arising from the Xth N if the initial symptoms of hoarseness and dysphagia had been considered. The prolonged duration of symptoms without earlier signs of increased intracranial pressure makes this diagnosis somewhat doubtful. The diagnosis of a carotid body tumor with invasion of the angle could not have been made in the absence of a palpable mass in the neck. The site of origin of this tumor can be only a matter of conjecture, the most plausible explanation is that it originally arose from displaced fragments of chromaffin cells in or in the neighborhood of the jugular foramen, producing early unilateral pressure on the glossopharyngeal and vagus nerves. The slow growth into the cranium explains the late appearance of other neurologic manifestations.

3 Malignant melanoma

Case report J B, W M, 62, Hist No 130428—Admitted Feb 14, 1938, with the history that four months previously he had developed pronounced numbness in the left hand which lasted for three weeks

and reappeared three weeks before admission. Diplopia, sudden loss of hearing on the right, dizziness in change of position of the head, and staggering to the right on walking had been present for four weeks. No headaches. On admission the physical examination was negative. The neurological examination showed a rhythmical horizontal nystagmus, weakness of the left side of the face, peripheral in type, total nerve deafness, right, absence of vestibular response on the right, ataxia of the right arm and astereognosis in left hand. Operation (Feb 15) ventricular air injection performed. Ventricles were normal. Unilateral cerebellar exploration performed on the right side. At the recess a dark reddish-brown tumor extending from the VIIIth to the Xth N was seen and did not seem to infiltrate either the cerebellum or brain stem. Tumor was shelled out completely. Weight 3.4 gms. Histological diagnosis metastatic malignant melanoma. The patient was discharged in apparently good condition, but died suddenly at home three months after operation. No autopsy performed.

Comments. The rapidity of symptom in quite an elderly individual should have made the diagnosis of a metastatic lesion at the cerebello-pontile recess quite obvious, even in the absence of clinical evidence of a primary source. The sensory changes in the left hand in the absence of evidence of pressure of the tumor on the brain stem at operation point to a small metastasis in the right parietal lobe in spite of the normal air studies. The late appearance of hearing difficulties would exclude a tumor arising from the VIIIth N.

CONCLUSIONS

- 1 The age of onset and duration of symptoms are the most important points in the differential diagnosis of tumors at the cerebello-pontile recess.

- 2 In the first two decades of life we have shown that the most common tumors are the unilateral acoustic neurinomas with peripheral neurofibromatosis, the bilateral acoustic neurinomas and abscesses. The average duration of symptoms of ten, five and one years respectively help in their differential diagnosis.

- 3 Gliomas at the recess occur predominantly between twenty and thirty years of age with a duration of symptoms varying from one to three years.

4 Between thirty and forty years of age the unilateral acoustic neurinomas, without peripheral neurofibromatosis, and the cholesteatomas are most commonly found. The former occur in the late 30's with an average duration of symptoms of four and a half years, while the latter start to manifest themselves in the early 30's with an average duration of symptoms of seven years.

5 The meningiomas have the same average duration of symptoms as the unilateral acoustic neurinomas but they appear mostly between forty and fifty years of age.

6 After the age of fifty, tumors at the cerebellopontile recess are uncommon.

7 There is no specificity in chronology of symptoms. Perhaps the only differential point is that most cases of acoustic neurinomas initiate their symptoms with auditory and/or labyrinthine disturbances, but we have seen that this is also true, although in a lesser degree, of the meningiomas and gliomas.

8 When the initial symptom is characterized by *tic douloureux* or other symptoms referable to the Vth N we should suspect either a cholesteatoma or a neurinoma arising from the Vth N.

9 The neurologic examination shows the same variation in signs in different tumors at the recess except in the cholesteatomas in which VIIIth N involvement is rare while involvement of the Vth N is quite common.

10 When X-rays of the base of the skull show erosion of the *porus acusticus* we must conclude that we are dealing with an acoustic neurinoma, as it is present in fifty per cent of these tumors and rarely in the rest.

SUMMARY

1 Two hundred and five cases of tumors of the cerebellopontile recess, verified at operation at The Johns Hopkins Hospital in a period of twenty years, have been analyzed.

2 Their main differential characteristics have been discussed.

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EFFECT OF PARA-AMINOHIPPURIC ACID ON SODIUM THIOSULFATE DETERMINATIONS IN RENAL CLEARANCE STUDIES

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In beginning a study of the renal blood flow in patients with congenital cyanotic heart disease, the clearance of para-aminohippuric acid was used for the determination of effective renal plasma flow and the functional capacity of the tubular excretory system (1) Sodium thiosulfate clearance was employed to measure glomerular filtration Thiosulfate clearance has recently been established as an accurate estimate of glomerular filtration by Gilman, Newman and co-workers (2, 3) Its advantage over inulin and mannitol clearances lies in the ease and accuracy of the chemical determination of thiosulfate Simultaneous clearances of para-aminohippuric acid (PAH) and sodium thiosulfate were carried out according to the general technique outlined by Homer Smith and associates (1)

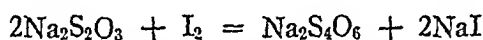
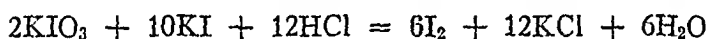
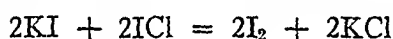
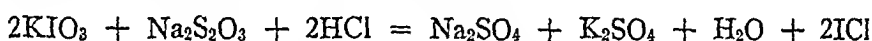
It was noted in these clearances that the determination of sodium thiosulfate in serum filtrates containing high concentrations of PAH gave unsatisfactory checks in duplicate aliquots Satisfactory checks were obtained with low concentrations of PAH and in serum blanks containing no PAH

It was also observed that the second thiosulfate determination in duplicate aliquots of the filtrates containing excessive PAH gave a value consistently higher than the first The only variant in the determination of the duplicates was the length of time of acidification before titration

These observations prompted an investigation of the effects of (1) varying concentrations of PAH, and (2) varying time intervals of acidification, on sodium thiosulfate titration values in aqueous, serum and plasma solutions of known concentration

METHODS

Para-aminohippuric acid was determined by the method of Smith, Finkelstein, Aliminosa, Crawford and Graber (1) Sodium thiosulfate was determined by the "indirect" method recommended by Newman, Gilman and Philips (3) for the low thiosulfate levels found in serum. In this procedure proteins are precipitated with sodium tungstate and sulphuric acid. After centrifugation a 10 ml aliquot of the supernatant fluid is added to 10 ml of 0.01 normal potassium iodate solution and acidified with 2 ml of 2 normal hydrochloric acid. The mixture is allowed to stand for at least five minutes. Then 2 ml of freshly prepared 10 per cent potassium iodide are added. The liberated iodine is immediately titrated with 0.01 normal sodium thiosulfate, with the addition of a few drops of 1 per cent fresh starch solution as indicator when the iodine color fades. According to Newman et al (3) the reactions involved are the following



The formula for the calculation of concentration in milligrams per cent is

$$\begin{aligned} \text{Standardizing titration} - \text{unknown titration} &\times \frac{1.58}{8} \\ &\times \frac{10}{\text{Standardizing titr}} \times \frac{100 \times 10}{\text{cc of filtrate aliquot used}} \end{aligned}$$

PREPARATION OF SOLUTIONS

In this series of experiments a stock 100 mg per cent sodium thiosulfate solution (156.9 mg $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ and 10 mg Na_2CO_3 dissolved in 100 ml distilled water) and a stock 750 mg per cent para-aminohippuric acid solution (375 mg para-aminohippuric acid dissolved in 50 ml distilled water with the aid of sodium carbonate) were prepared.

In the plasma and serum experiments two variations were employed. In the first, 35 ml of serum or citrated plasma and 10 ml of the 100 mg per cent sodium thiosulfate solution were mixed with either 5 ml of the 750 mg per cent para-

aminohippuric acid solution, 5 ml of some dilution of this solution, or with 5 ml of distilled water in 50 ml volumetric flasks. Serum or citrated plasma blanks consisted of 35 ml of serum or citrated plasma and 15 ml distilled water. To an aliquot of these stock serum or citrated plasma solutions was added seven volumes of water, one volume of 5 per cent sodium tungstate solution and one volume of one-third normal sulphuric acid solution. After centrifugation and filtration, aliquots were used for sodium thiosulfate determinations. In another aliquot of the stock serum or citrated plasma proteins were precipitated with acid cadmium sulfate and sodium hydroxide solutions and the filtrate was used to estimate the PAH level. In the second variation suitable portions of dilutions of the stock solutions were added to serum and oxalated plasma, whose protein was then precipitated with sodium tungstate and sulphuric acid solutions as above. Thus in both cases sodium thiosulfate and PAH were present at the time of protein precipitation and were not added afterwards.

In the case of aqueous solutions, 10 ml of the 100 mg per cent sodium thiosulfate solution were diluted to 50 ml in a volumetric flask and 25 ml of this were diluted to 250 ml, or 5 ml of the stock 100 mg per cent sodium thiosulfate solution were diluted directly to 250 ml in a volumetric flask. PAH was either added before diluting by using a suitable quantity of the stock solution or the necessary amount of powder was weighed out and dissolved in the diluted sodium thiosulfate.

The 0.01 N potassium iodate solution was obtained by diluting the 0.1 N stock solution (3.567 grams potassium iodate dissolved in one liter distilled water) ten-fold on the day of use. The 10 per cent potassium iodide solution was prepared in small amounts, was kept in a brown bottle in an ice bath and was used not longer than one hour after preparation. The starch solution was prepared freshly each day and was centrifuged before use. The sodium thiosulfate solution used for the titration of the liberated iodine was made more than 0.01 N and its true concentration was determined with the 0.01 N potassium iodate. A 10 ml micro burette, graduated to fiftieths of a milliliter, was employed and readings were made to hundredths of a milliliter. All titrations were performed at room temperature in the absence of direct sunlight. An interval timer was used to measure the time between the addition of the acid and of the potassium iodide which was immediately followed by titration of the liberated iodine.

EXPERIMENTAL PROCEDURES AND RESULTS

Control experiments were set up which used water, serum and plasma solutions of sodium thiosulfate in a concentration of 20 mg per cent. This level represents the desirable range of thiosulfate concentration for the measurement of glomerular filtration rate in man (4). Equivalent solutions of sodium thiosulfate were prepared containing low, intermediate and high concentrations of PAH. Titrations in duplicate (occasionally in triplicate) of sodium thiosulfate in filtrates of these

solutions were then performed after intervals between acidification and titration of 1, 2, 3, 5, 15, 30 and 60 minutes. Correction was made for the serum or plasma blank titration in each instance (3).

A few experiments were conducted along exactly similar lines with sodium thiosulfate in 40 and 60 mg per cent concentration.

The results of these experimental determinations are presented in Table I in summary form. For each set of observations there is presented the average determination, the range and the number of determinations. In 105 determinations of the aqueous controls (20 mg per cent sodium thiosulfate solutions containing no PAH) the average is 19.90 mg per cent with a range of 19.3 to 20.7 mg per cent. The average for the aqueous 40 mg per cent sodium thiosulfate control containing no PAH (20 determinations) is 39.77 mg per cent with a range of 39.4 to 40.4 mg per cent. The average for the aqueous 60 mg per cent sodium thiosulfate controls containing no PAH (18 determinations) is 59.32 mg per cent with a range of 58.8 to 60.0 mg per cent.

It will be assumed that if the average of a set of determinations falls outside of a range of two per cent greater to two per cent less than the respective control average, there is a significant difference between that set and the control set. For 20 mg per cent sodium thiosulfate this range is 19.5 to 20.3 mg per cent, for 40 mg per cent, 39.0 to 40.6 mg per cent, for 60 mg per cent, 58.1 to 60.5 mg per cent.

In Figure 1 the effect of high and low concentrations of PAH on 20 mg per cent sodium thiosulfate determinations is plotted as a function of the time between acidification and titration. From the graph it is seen that in the presence of high concentrations of PAH the apparent sodium thiosulfate levels increase rapidly with time. This occurs in water, serum and plasma solutions. With low concentrations of PAH this increase in apparent sodium thiosulfate levels is manifested to a limited extent in plasma solutions but is not observed in serum and water solutions. The use of plasma is not recommended, since oxalate and citrate appear to augment the false elevation.

Essentially the same results are obtained with 40 and 60 mg per cent sodium thiosulfate in aqueous and serum solutions containing high concentrations of PAH.

The false elevation of aqueous sodium thiosulfate levels increases slightly with increased sodium thiosulfate concentration when the

TABLE I

The effect of varying the concentration of para-aminoluppuric acid and varying the interval between acidification and titration on the calculated concentration of sodium thiosulfate

TRUE MG % SODIUM THIO- SULFATE	MG % PAH	MEDIA	CALCULATED MG PER CENT SODIUM THIOSULFATE USING VARYING INTERVALS BETWEEN ACIDIFICATION AND TITRATION							
			1 min	2 min	3 min	5 min	15 min	30 min	60 min	
20 0	0	Water	19 88	19 78	19 92	19 85	20 05	19 88	19 98	Aver
			20 7	20 2	20 5	20 4	20 2	20 2	20 2	Max
			19 4	19 5	19 3	19 4	19 7	19 6	19 5	Min
			(24)	(5)	(13)	(28)	(12)	(11)	(12)	N
20 0	2 18 2 76 4 11	Water	20 17		20 03	19 83	20 06	20 59	20 56	Aver
			20 6		20 4	20 2	20 6	22 0	21 2	Max
			19 8		19 7	19 5	19 4	19 8	19 6	Min
			(6)		(6)	(6)	(6)	(7)	(8)	N
20 0	75* 75* 75*	Water	20 28		21 20	22 09	23 83	26 15	28 13	Aver
			20 7		21 6	22 6	24 4	26 8	29 4	Max
			19 5		20 7	21 6	23 2	25 4	27 0	Min
			(6)		(6)	(7)	(6)	(6)	(6)	N
20 0	0	Plasma	19 70		20 03	20 26	20 27	20 53†	20 73†	Aver
			20 6		20 6	20 8	21 1	21 1	21 5	Max
			19 0		19 3	19 7	19 8	20 1	19 9	Min
			(6)		(6)	(8)	(6)	(6)	(6)	N
20 0	2 27 2 85	Plasma	19 38		20 20	20 97	20 50	21 30†	21 65†	Aver
			20 0		20 6	21 4	21 3	21 9	22 5	Max
			18 8		19 6	20 6	19 6	20 7	20 9	Min
			(4)		(4)	(6)	(4)	(4)	(4)	N
20 0	69 5 74 0 75*	Plasma	19 97		21 72	22 26	24 67	27 67†	29 48†	Aver
			20 5		23 0	22 6	25 7	28 1	30 4	Max
			19 0		20 9	21 5	23 5	26 1	28 0	Min
			(6)		(6)	(8)	(6)	(6)	(6)	N
20 0	0	Serum	19 22	19 52	19 20	19 98	19 70	19 60	19 90	Aver
			19 8	20 0	19 3	20 4	19 9	19 7	19 9	Max
			18 3	19 2	19 1	19 2	19 5	19 5	19 9	Min
			(9)	(5)	(3)	(9)	(2)	(2)	(2)	N

TABLE I—*Continued*

TRUE MG % SODIUM THIO- SULFATE	MG % FAH	MEDI	CALCULATED MG PER CENT SODIUM THIOSULFATE USING VARYING INTERVALS BETWEEN ACIDIFICATION AND TITRATION							
			1 min	2 min	3 min	5 min	15 min	30 min	60 min	
20 0	2 41	Serum	19 15	19 46	19 53	19 90	19 80	20 00	20 60	Aver
	2 94									
	2 96		19 6	19 8	20 0	20 8	20 0	20 2	20 7	Max
	3 02		18 4 (12)	19 2 (7)	19 1 (6)	19 2 (12)	19 7 (4)	19 9 (4)	20 3 (4)	Min N
20 0	72 2	Serum	19 69	20 24	20 43	22 12	23 70	25 40	27 70	Aver
	73 1									
	73 6		20 0	20 8	20 5	22 8	23 9	25 5	28 1	Max
			19 2 (9)	19 8 (5)	20 3 (3)	20 9 (9)	23 5 (2)	25 3 (2)	27 3 (2)	Min N
40 0	0	Water	39 64	39 60	39 8	39 92		39 4	39 84	Aver
			40 2	39 8		40 4			40 4	Max
			39 4 (5)	39 4 (2)	(1)	39 5 (6)		(1)	39 4 (5)	Min N
40 0	73 2	Water	39 74	40 37	41 61	42 59	47 22	50 08	51 64	Aver
	75*									
	75*		40 2	40 6	42 5	43 3	47 6	50 6	53 9	Max
			39 2 (7)	40 0 (6)	41 0 (7)	41 8 (9)	46 7 (6)	49 2 (6)	46 3 (7)	Min N
40 0	0	Serum	39 53	39 87	40 30	40 50	38 7	39 5	40 3	Aver
			39 6	40 0	40 4	41 4				Max
			39 4 (3)	39 8 (3)	40 2 (2)	40 2 (6)	(1)	(1)	(1)	Min N
40 0	75*	Serum	40 20	41 47	42 10	43 50	49 7	51 3	56 10	Aver
			40 6	42 0	42 2	43 5			56 4	Max
			39 8 (3)	41 2 (3)	42 0 (2)	43 5 (2)	(1)	(1)	55 8 (2)	Min
60 0	0	Water	59 27	59 40	59 40	59 47	59 20	59 30	59 10	Aver
			59 6	59 8	59 6	60 0	59 2	59 6	59 4	Max
			58 8 (3)	59 2 (3)	59 0 (3)	58 8 (3)	59 2 (2)	59 0 (2)	58 8 (2)	Min N

TABLE I—*Concluded*

TRUE MG % SODIUM THIO- SULFATE	MG % PAH	MEDIA	CALCULATED MG PER CENT SODIUM THIOSULFATE USING VARYING INTERVALS BETWEEN ACIDIFICATION AND TITRATION							
			1 min	2 min	3 min	5 min	15 min	30 min	60 min	
60 0	75*	Water	59 87	60 47	61 00	62 47	67 90	72 10	76 90	Aver
			60 0	60 8	61 2	62 8	68 2	72 2	77 6	Max
			59 8	60 2	60 6	62 0	67 6	72 0	76 2	Min
			(3)	(3)	(3)	(3)	(2)	(2)	(2)	N
60 0	0	Serum	59 60	59 93	60 30	60 47	59 5	60 1	60 5	Aver
			60 4	60 2	60 6	60 8				Max
			58 9	59 6	60 0	60 0				Min
			(3)	(3)	(2)	(6)	(1)	(1)	(1)	
60 0	75*	Serum	60 20	61 47	62 90	64 70	69 0	74 5	80 00	Aver
			60 6	61 8	63 0	64 8			80 1	Max
			60 0	61 2	62 8	64 6			79 9	Min
			(3)	(3)	(2)	(2)	(1)	(1)	(2)	N

Aver = average, Max = maximum, Min = minimum, N = number of determinations, Min = minute(s)

* Weighed All other concentrations were determined colorimetrically

† Opalescent when titrated

PAH concentration is maintained at 75 mg per cent. However when the sodium thiosulfate concentration is maintained at 20 mg per cent while the PAH concentration is varied, the false elevation is nearly proportional to the PAH concentration in the range of 35 to 75 mg per cent. This false elevation amounts to 0.03 mg per cent sodium thiosulfate for each mg per cent PAH present in the undiluted material.

An attempt was made to determine the minimal concentration of PAH required to produce a false elevation of the aqueous 20 mg per cent sodium thiosulfate level. The false elevation was equivocal at 25 mg per cent PAH but was definite at 35 mg per cent PAH using a 5 minute interval between acidification and titration.

COMMENT

Since the false elevation of the sodium thiosulfate level in the presence of high concentrations of PAH increases with the time that

elapses between the addition of acid (reaction I above) and the addition of potassium iodide (reactions II and III above), a reaction must occur between PAH and either potassium iodate, iodine monochloride or both. Regardless of the specific nature of the reaction, it gives rise to a decreased amount of liberated iodine and therefore a decrease in the amount of sodium thiosulfate required for titration, thus indicating an incorrectly high concentration of sodium thiosulfate.

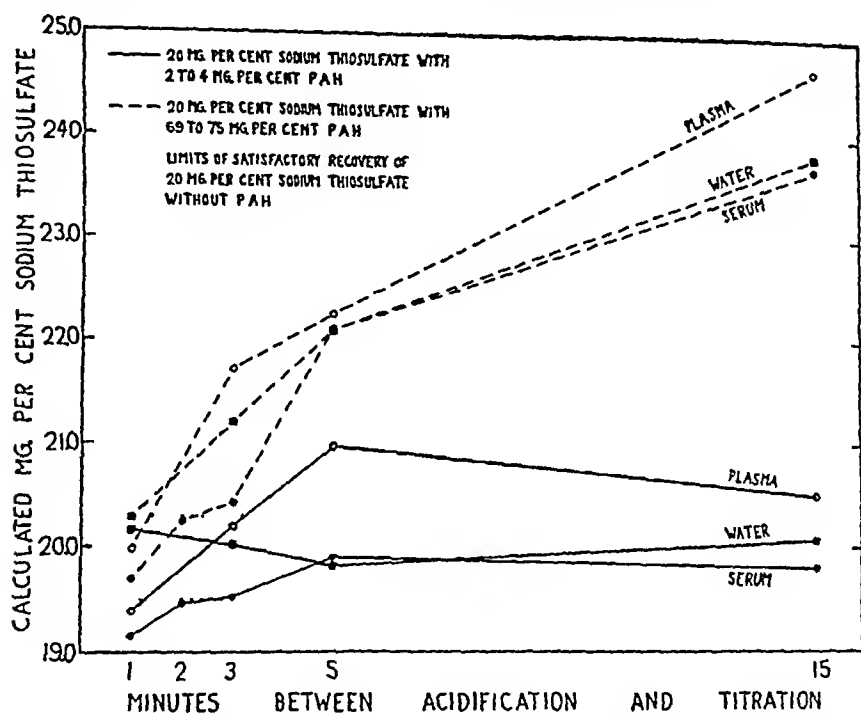
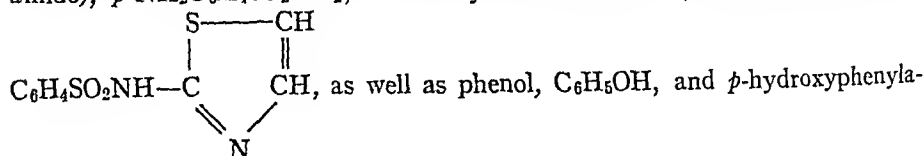


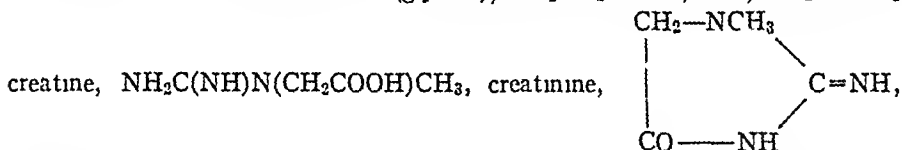
FIG 1 THE EFFECT OF HIGH AND LOW CONCENTRATIONS OF PAH ON SODIUM THIOSULFATE DETERMINATIONS PLOTTED AS A FUNCTION OF TIME BETWEEN ACIDIFICATION AND TITRATION

In addition to para-aminohippuric acid, $p\text{-NH}_2\text{C}_6\text{H}_4\text{CONHCH}_2\text{COOH}$, several chemically similar compounds at approximately the same concentration produce a similar false elevation of the sodium thiosulfate level when the latter is measured by this method. These compounds include aniline hydrochloride, $\text{C}_6\text{H}_5\text{NH}_2 \text{ HCl}$, para-aminobenzoic acid, $p\text{-NH}_2\text{C}_6\text{H}_4\text{COOH}$, para-aminobenzenesulfonic acid (sulfanilic acid), $p\text{-NH}_2\text{C}_6\text{H}_4\text{SO}_3\text{H}$, para-aminobenzenesulfonamide (sulfanilamide), $p\text{-NH}_2\text{C}_6\text{H}_4\text{SO}_2\text{NH}_2$, 2-sulfanilyl aminothiazole (sulfathiazole), $p\text{-NH}_2\text{C}_6\text{H}_4\text{CONHCH}_2\text{COOH}$, as well as phenol, $\text{C}_6\text{H}_5\text{OH}$, and p -hydroxyphenyl-



lanine (tyrosine), $p\text{-HOC}_6\text{H}_4\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$ When one of these aromatic compounds at the same concentration is subjected to the entire procedure in the absence of sodium thiosulfate, a titration value identical with that of acidified potassium iodate alone is obtained, even if a sixty minute interval between acidification and titration is employed. This would indicate that the side reaction is not oxidation by potassium iodate, but rather some reaction involving iodine monochloride which is produced when sodium thiosulfate is present. In the case of phenol and tyrosine halogenation of the ring, analogous to bromination of phenol, is the most likely reaction. Nascent iodine reacts slowly with PAH. However, halogenation of the ring is probably not the reaction in the case of the amines because both acetanilide, $\text{CH}_3\text{CONHC}_6\text{H}_5$, and para-acetylaminohippuric acid, $p\text{-CH}_3\text{CONHC}_6\text{H}_4\text{CONHCH}_2\text{COOH}$, cause no false elevation of the thiosulfate levels although both compounds should halogenate. Similarly the substitution of chlorine for one of the hydrogens attached to the amine nitrogen group should not be prevented by acetylation of the amine. However, if the reaction is oxidation of the amine by iodine monochloride, acetylation should prevent this, just as acetylation prevents the oxidation of aniline when the latter is nitrated with concentrated nitric acid, a strong oxidizing agent.

No elevation of the true sodium thiosulfate level was observed with a similar concentration of aminoacetic acid (glycine), $\text{NH}_2\text{CH}_2\text{COOH}$, urea, NH_2CONH_2 ,



guanidine hydrochloride, $(\text{NH}_2)_2\text{CNH HCl}$ or glutamic acid, $\text{HOOC}(\text{CH}_2)_2\text{CH}(\text{NH}_2)\text{COOH}$

A chemical method is available for the determination of sodium thiosulfate in which no iodine monochloride is formed. This is the "direct" method given by Newman et al (3) for use with urine or the "iodide" method of Gilman et al (2) for plasma filtrates and urine. These procedures vary only in the amount of potassium iodate used, since in each the material to be titrated is alkalinized before the addition of the appropriate amount of potassium iodate solution, so that no iodine monochloride can be produced. Then potassium iodide and hydrochloric acid are added in that order. The liberated iodine is immediately titrated with sodium thiosulfate. When the titration is performed immediately, a multifold excess of PAH causes no elevation of the true concentration of sodium thiosulfate. However, since this method is only an eighth as sensitive as the indirect method, it is not suitable for such low concentrations of sodium thiosulfate as 20 mg per cent.

PAH is reported to cause false elevations of mannitol determination when these substances are used together in clearances. Early in 1947 Barker and Clark (5) observed an elevation of 0.25 to 0.30 mg per cent in the true mannitol level for each mg per cent PAH present in low, intermediate and high concentrations. The method for determining mannitol involves the oxidation of mannitol by a known excess of acid potassium periodate solution with the aid of heat. The potassium

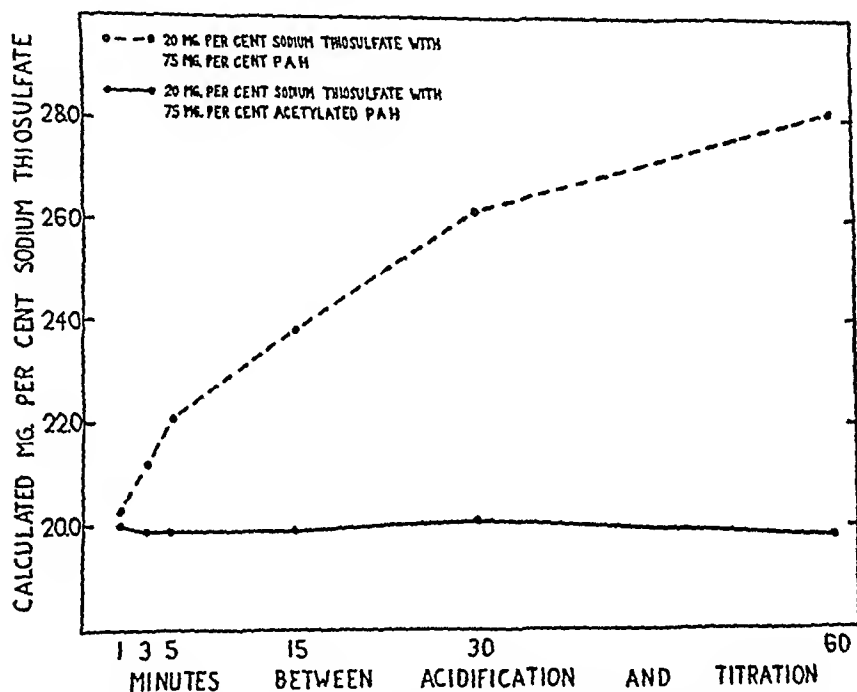


FIG 2 COMPARISON BETWEEN THE EFFECTS OF PAH AND ACETYLATED PAH ON SODIUM THIOSULFATE DETERMINATIONS

iodate which is formed plus the residual potassium periodate is then determined by thiosulfate titration. Barker and Clark attributed the false elevation of mannitol values to the oxidation of PAH by periodate.

These authors developed a modified method for mannitol determinations in which PAH is acetylated and in this manner they are able to avoid false elevations of mannitol. Similarly acetylated PAH causes no false elevation of sodium thiosulfate values as illustrated in Figure 2. An attempt was made to introduce acetylation of PAH into the in-

direct procedure for thiosulfate determination. However, under the conditions employed in several experiments only irregular partial recovery was obtained with known sodium thiosulfate solutions containing high concentrations of PAH.

As suggested by Newman (4) cooling of the aliquot of serum filtrate in an ice bath for at least one hour prior to the addition of potassium iodate and hydrochloric acid permits the use of a five minute interval at room temperature when the PAH concentration is 75 mg per cent. In the absence of PAH cooling does not appear to appreciably affect either the determination of the serum blank or the determination of sodium thiosulfate in serum filtrates. As would be expected, warming of the aliquot greatly increases the false elevation of the sodium thiosulfate level.

If the entire procedure, with the exception of the titration of the liberated iodine, is performed in the dark the same false elevation is observed as when light is present. In an effort to reduce the PAH concentration of the aliquot to a level at which it would not interfere, threefold dilution of the aliquot was made before the addition of potassium iodate and hydrochloric acid. After five minutes the iodine was liberated and immediately titrated. The false elevation is thereby diminished by approximately one mg per cent. With sixfold dilution no further depression of the false elevation is observed. Two-fold dilution does not diminish the false elevation at all.

CONCLUSIONS

1 In the presence of high concentrations of PAH false elevations of sodium thiosulfate levels occur when the "indirect" method of Newman et al is used.

2 These false elevations increase with the time interval between acidification and titration in the "indirect" method of thiosulfate determination.

3 In order to obtain accurate values for sodium thiosulfate in the presence of PAH by this method the following factors must be observed:

- (1) With low PAH concentrations the time interval should be,
 - (a) Five minutes for serum filtrates
 - (b) Three minutes for citrated and oxalated plasma filtrates

- (2) With high PAH concentrations the time interval should be,
 - (a) One minute for uncooled serum and plasma filtrates
 - (b) Five minutes for thoroughly chilled serum filtrates
- 4 Acetyl-PAH does not cause false elevations of sodium thiosulfate levels, and a satisfactory method for acetylation of PAH in this procedure is needed

SUMMARY

The effect of para-aminohippuric acid on sodium thiosulfate determinations in renal clearance studies has been assessed. False elevations of thiosulfate are observed in the presence of PAH under certain conditions. Investigation of these elevations is detailed and suggestions are made for their avoidance.

We are indebted to Dr Elliot V Newman, Dr J Logan Irvin and Dr Richard J Bing whose helpful advice and criticism aided greatly in this project.

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THERAPEUTIC EVALUATION OF ISO-PAR¹ IN OTITIS EXTERNA

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The search for an antibacterial, antipruritic agent efficacious in the treatment of otitis externa has been a continuous one. The multiplicity of medications now in use is sufficient proof of this statement. The development of Iso-Par has provided us with a valuable addition for therapy of this obstinate condition. This article reports the results obtained with this ointment in acute and chronic otitis externa. No attempt was made at selection of patients nor was emphasis placed upon the bacteriological findings. Instead, the clinical picture and course were the determining factors in evaluation.

Reardon (1) found that the use of Iso-Par was far superior to any other type of treatment he had used previously in his experience with otitis externa in the army. During a period of two years in the tropics, he was able to observe several hundred patients with disease. He concluded that the ointment was definitely beneficial, non-toxic, locally anesthetic, and bactericidal in its action.

Iso-Par is a suspension of paraffinic acids in a base of lanolin and petrolatum. Although in vitro tests are inconclusive, definite bactericidal and fungicidal properties may be assigned to this ointment.

The routine office treatment was simple and expedient. With alcohol soaked applicators the ear canal was carefully cleansed of all debris and discharge. A thin coating of ointment was then applied. This procedure was repeated in the clinic during the acute phase of the infection as often as necessary. Later the patient was instructed in the application of the ointment at home. Supportive symptomatic

* Iso-Par is a mixture of water insoluble isoparaffinic acids partially neutralized with isocetyl hydroxybenzyl-dialiphatic amines. Iso-Par unguentum contains 17 per cent of Iso-Par and 4 per cent of titanium dioxide in an ointment base consisting of beeswax, cetyl alcohol, lanolin, and petroleum. U N R, 1947 p 90

care in the form of heat, aspirin, and codeine was prescribed as indicated

Subjectively, the annoyance of itching and the relief of pain were noticed usually after the initial application of the ointment. With the abatement of these symptoms it was much easier to care for the affected ear on the succeeding visits. In those patients suffering from recurrent infections, the prophylactic use of Iso-Par once a week was advised.

Forty-one patients are considered in the present report. Many others were treated but did not return to the clinic. Twenty-three acute and eighteen chronic cases of otitis externa are summarized. A case was classed as chronic if symptoms had persisted over three months or history indicated the infection was a recurrent one. For study, a division was made according to the severity of the infection. Thus, the mild cases were those patients who exhibited some itching and occasional discharge from the ear. The patients who had findings of moderate discharge, redness, edema, and complained of pain were considered moderately severe infections. All those with the above findings present, plus evidence of a cellulitis of the soft tissues of the ear canal and surrounding area, were classed as severe.

In the accompanying table, it will be seen that the patients' ages varied from eleven to seventy years. Except for two circumscribed infections, the usual picture was that of diffuse involvement. Twenty-nine, or almost two-thirds of the cases, were of the "moist" variety, in contrast to the dry or non-eczematous type seen in the remainder. The former are usually most resistant to therapy.

Following the regime as outlined, it was possible to class twenty-five or sixty per cent as cured, ten or twenty-four per cent as improved, and six or only sixteen per cent as unimproved. No average length of treatment period could be established. Some patients required constant care over a two month period before finally clearing, while others required therapy for only five to seven days.

The results of the series reported here are in agreement with the impressions gained by Reardon. Iso-Par has proven to be of definite value in the treatment of otitis externa. No sensitization to the drug was seen. It relieved pain and pruritis by its local action.

Summary of Results with Iso-Par in Otitis Externa

PATIENT	AGE	RACE SEX	ACUTE	CHRONIC	MOIST	DRY	UNILATERAL	BILATERAL	PREVIOUS R	DAYS ISO PAR	CURED	IMPROVED	UNIMPROVED	DEGREE
1 AH	47	cf	No	Yes	Yes	No	No	Yes	No	14	Yes	No	No	Sev
2 HC	43	wm	Yes	No	Yes	No	No	Yes	Yes	45	No	No	Yes	Sev
3 AC	60	wf	No	Yes	Yes	No	No	Yes	No	12	No	Yes	No	Sev
4 CM	53	wm	Yes	No	No	Yes	No	Yes	No	14	Yes	No	No	Mild
5 WO'M	31	wm	Yes	No	Yes	No	Yes	No	No	12	No	Yes	No	Sev
6 AP	36	cm	Yes	No	Yes	No	Yes	No	No	10	Yes	No	No	Mild
7 MA	25	wf	Yes	No	Yes	No	Yes	No	No	7	Yes	No	No	Mod
8 BH	20	wf	No	Yes	Yes	No	No	Yes	No	21	Yes	No	No	Sev
9 EM	47	wm	Yes	No	Yes	No	Yes	No	No	3	No	No	Yes	Sev
10 EC	45	wf	Yes	No	Yes	No	No	Yes	No	8	No	No	Yes	Sev
11 AR	27	wf	Yes	No	No	Yes	No	Yes	No	60	Yes	No	No	Sev
12 JK	23	wm	No	Yes	Yes	No	No	Yes	Yes	30	No	Yes	No	Sev
13 LA	25	cm	Yes	No	Yes	No	Yes	No	Yes	4	No	No	Yes	Mod
14 JM	19	wm	Yes	No	Yes	No	Yes	No	No	24	Yes	No	No	Sev
15 DT	8	wm	Yes	No	Yes	No	Yes	No	No	6	Yes	No	No	Sev
16 DF	44	cf	Yes	No	Yes	No	Yes	No	No	13	Yes	No	No	Sev
17 EJ	38	wf	No	Yes	No	Yes	No	Yes	No	5	No	No	Yes	Mod
18 EE	32	wf	Yes	No	Yes	No	Yes	No	Yes	60	No	Yes	No	Sev
19 SF	68	wf	No	Yes	No	Yes	No	Yes	No	21	Yes	No	No	Mod
20 AS*	26	wf	No	Yes	Yes	No	No	Yes	No	14	Yes	No	No	Mod
21 AC	24	cf	Yes	No	Yes	No	No	Yes	No	3	Yes	No	No	Mod
22 PW	33	wf	No	Yes	Yes	No	No	Yes	No	7	No	Yes	No	Mod
23 LJ	63	wf	No	Yes	No	Yes	No	Yes	No	5	Yes	No	No	Mod
24 WJ	20	wm	No	Yes	No	Yes	No	Yes	Yes	4	Yes	No	No	Mild
25 LB	36	wf	No	Yes	Yes	No	No	Yes	Yes	7	No	Yes	No	Sev
26 MT	70	wm	Yes	No	Yes	No	No	Yes	No	6	Yes	No	No	Mod
27 AM	22	wm	Yes	No	Yes	No	Yes	No	No	7	Yes	No	No	Mod
28 AW	11	wf	Yes	No	Yes	No	Yes	No	No	21	No	No	Yes	Mod
29 SV	11	wf	No	Yes	Yes	No	Yes	No	Yes	9	Yes	No	No	Mild
30 PC	59	wm	No	Yes	Yes	No	No	Yes	Yes	67	Yes	No	No	Mod
31 MH	68	wf	Yes	No	No	Yes	No	Yes	No	5	Yes	No	No	Mild
32 TM	15	cf	No	Yes	Yes	No	No	Yes	Yes	13	Yes	No	No	Mod
33 EB	44	cf	Yes	No	No	Yes	No	Yes	No	7	Yes	No	No	Mild
34 MJ	53	wf	No	Yes	No	Yes	No	Yes	No	35	No	Yes	No	Mod
35 PH	56	wf	No	Yes	No	Yes	Yes	No	No	4	Yes	No	No	Mild
36 GR	14	cf	Yes	No	Yes	No	Yes	No	No	9	Yes	No	No	Sev
37 MH	14	wf	Yes	No	Yes	No	No	Yes	Yes	7	Yes	No	No	Mod
38 EB*	57	wm	Yes	No	No	Yes	Yes	No	Yes	7	Yes	No	No	Sev
39 LT	33	cf	No	Yes	Yes	No	No	Yes	No	2	No	Yes	No	Mod
40 SL	12	wf	Yes	No	Yes	No	Yes	No	Yes	9	No	Yes	No	Sev
41 CD	46	cm	No	Yes	No	Yes	No	Yes	No	60	No	Yes	No	Mild
			23	18	29	12	16	25	12		25	10		8 Mild 16 Mod 17 Sev — 41

* Circumscribed lesions

A high percentage of cures was effected within a minimum of time
It should be considered a valuable aid in therapy of otitis externa

REFERENCE

- 1) REARDON, WM T Use of Iso-Par Ointment in the Treatment of Otitis Externa
Arch Otol , 45 294, March 1947

PREFRONTAL LOBOTOMY FOR THE RELIEF OF INTRACTABLE PAIN

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Pain, as a subjective experience, is a complex phenomenon. Psychologists still express some disagreement about whether pain is a sensation or an emotion. Looked upon as a sensation it has, in common with other sensations, a threshold, the possibility of localization, as well as reference to a stimulus. Unlike the other sensations, such as vision and touch, however, pain is involved much more closely with the center of personality and has a good deal in common with some of the emotions such as fear and guilt. Studies on pain in the physiological laboratory have almost entirely, but necessarily, been confined to the so-called "objective" data referable to thresholds, transmission of impulses, fatigability, and so on. It has thus far not been possible for physiological methods to differentiate between the purely sensory component of pain and the "unpleasantness" or the "reaction to pain" of the emotional element. This was placed in the realm of the psychologist (1).

Relief of pain is one of the primary concerns of the physician. All analgesic drugs, of course, have their place in the treatment of the occasional mild pain or pain of short duration, but where one is dealing with chronic or persistent pain the simpler analgesics are ineffective, while the narcotics carry the grave danger of addiction. Under these circumstances, the surgeon is frequently called upon to break the pathway of pain on the physiological level. This involves nerve-block, root section, chordotomy, or even resection of the sensory cerebral cortex. This surgical treatment, however, does not always produce the desired relief, either because of previous addiction to drugs, or because of the essentially subjective nature of the symptoms. It is important to bear in mind the difference between mere perception of pain and reaction to pain. The latter includes not only certain autonomic reactions such as sweating or tachycardia, but also the

emotional and attitudinal reactions, such as anxiety, fear, panic, or even prostration. It is failure to control the latter that makes so many surgical measures for relief of pain ineffective.

Considerable progress has been made in the treatment of intractable pain by prefrontal lobotomy. Freeman and Watts (2) noted that in their series of patients with nervous and mental disease upon whom prefrontal lobotomies were done, there were a number who had complained of unbearable pain. It is important that the complaints of these patients seemed to be out of all proportion to the painfulness of the condition giving rise to the complaints. After operation not only were the complaints no longer heard, but nervous tension, anxiety and apprehension disappeared. This experience led to the use of prefrontal lobotomy in patients who had intractable pain, but no nervous or mental disease which in itself justified lobotomy. Poppen (3), Sargent (4), and others have reported striking relief of painful conditions which had resisted other forms of surgical therapy, such as carcinoma, tabes dorsalis, and phantom limb pain.

Our own experience thus far has been limited to patients with incurable cancer, all individuals whose life expectancy was relatively short. In these patients it was clear that there was great pain, but nervous tension, apprehension and fear of pain all seemed to be factors of greatest importance.

Case Reports It is interesting to consider the details in a few of our patients. I have picked out five which should be of interest.

Case 1 A A, WM, 62. Three years before admission this patient developed hematuria. A bladder tumor was removed suprapubically in 1943. Five or six months before admission to the Brady Institute in October, 1946, he again developed hematuria and more tumor was removed cystoscopically. From then on he lost appetite, lost 20 pounds in weight and developed a rather severe low back pain which grew steadily worse. When seen here a large bladder tumor was demonstrated on the right side. This was thought to represent an infiltrating carcinoma, grade C, inoperable. Deep X-ray therapy was instituted, but the patient continued to have severe pain in the back. The patient was given 12 mgm of morphine at first every four hours, later increased to every hour. About one month after his admission to the hospital he developed severe pain and swelling of the left leg and became bed-ridden. Inasmuch as this pain was unilateral and severe enough to cause increase of morphine administration to hourly intervals, a chordotomy was done. A satisfactory level of analgesia was obtained, but the patient began to complain more than ever of pain in his back and now his right leg. It was

necessary to continue hourly hypodermics as before. The patient was apprehensive, in a constant state of anxiety, anticipating both his pain and his hypodermics. Accordingly on January 8, 1947, three weeks after chordotomy, a prefrontal lobotomy was done. The patient became disoriented and confused and was incontinent both of urine and feces. These symptoms gradually improved. Six weeks after operation there were still a few aberrations, such as an apparent desire to get into other patients' beds, or to get into the tub for a bath and turn on the hot water without regard for burning himself. From the moment of operation it was clear that he either had no pain, or that it did not bother him. When asked whether he had pain he either denied it, or replied "Yes, but it doesn't bother me." Although he had received morphine or Schlesinger's solution every hour for nearly two months before lobotomy, he did not now require even an aspirin tablet. He was apathetic not only about the pain, but looked upon the hopelessness of his fate, and even impending death, with complete equanimity. This in spite of a previous state of anxiety and great apprehension. Only one glance at him sufficed to show that he was comfortable. He was no longer bed-ridden, but could walk about quite normally. His course after leaving the hospital on March 1, 1947, is unknown.

Case 2 T B, CM, 58. This patient was admitted to the Osler Medical Service on August 29, 1946, complaining of pain in the chest and back of two months' duration. Four days before his admission he developed weakness and numbness of his legs. Within twenty-four hours there was complete paralysis of the legs and anesthesia up to the umbilicus. He developed a cord bladder and fecal incontinence. On examination a carcinoma of the prostate could be palpated. Subsequent biopsy revealed a poorly differentiated carcinoma. Spinal puncture revealed a complete block. Because of the rapid course of the paralysis with fever and severe localized pain and tenderness at T6, a laminectomy was done with the hope of finding an epidural abscess, but none was disclosed. Widespread bony metastases were later discovered. The patient continued to have severe pain in his chest and he was placed on amidone in doses about equivalent to morphine. This gradually became ineffective and by early November he was taking as much as 32 mgm of morphine every two hours. Tubo-curare was also tried but was not significantly effective. The patient was quite apprehensive and asked for his medicine by the clock. On February 5, 1947, prefrontal lobotomy was carried out. He became confused and disoriented, but gradually regained recognition of his family. It is interesting to read the medical house-officer's comments of two weeks after operation: "Clinically Thomas is, of course, no better, and the appearance of his bed sores is appalling (he had, of course, had these before operation). Mentally, however, he is indeed a new man. He realizes that he is sick and that he is in pain from time to time, but it does not bother him one whit. He tells us that he feels just fine and I'm sure he means it." Because of apparent restlessness, two doses of sodium luminal were given in the seven weeks' interval between his operation and his death of generalized carcinomatosis.

Case 3 D L, WF 52. Two years before this patient's admission to the hos-

pital she began to have attacks of sore throat and difficulty in swallowing. In the fall of 1945 she went to another clinic where a biopsy of a tumor in the neck was taken and she was told she had a carcinoma of the larynx. X-ray therapy was given. In April, 1946, she was given further X-ray therapy and a tracheotomy was done. Three months prior to her first admission here she developed pains in the chest and paroxysmal painful coughing which was non-productive. The pain was constantly increased by deep respiration. There had been no loss in weight. Admission to the Surgical Service took place on November 24, 1946. She had a tracheotomy tube in place. No growth could be seen or felt in the neck. No tumor of larynx or bronchus could be found by bronchoscopy. X-ray showed a mass in the right lower lobe of the lung. On December 13, 1946, exploratory thoracotomy was performed and an inoperable tumor of the lung was found. The tumor mass had eroded through the diaphragm to involve the liver. There were large mediastinal nodes. The patient was discharged on December 30, 1946, with the diagnosis of inoperable carcinoma of the lung. Following discharge the patient gained a little weight, but had a frequent painful cough and almost constant complaint of pain in the lower chest. This spread up over the chest wall and began to involve the right arm. She was taking narcotics about every four hours, was quite tense and feared her cough. Pain gradually grew worse and she was readmitted on February 26, 1947. A week later prefrontal lobotomy was carried out. Following this she had no complaint of pain and no sedatives were required. On questioning at times she admitted having pain, but was obviously not uncomfortable. Interestingly enough, once her apprehension was removed, her cough nearly ceased altogether. The tracheotomy wound was closed. This patient showed no confusion or disorientation. She was the intelligent wife of an intelligent man. Before discharge from the hospital on March 19, 1947, he was asked if he noted any change in her personality. At first he said there was none, then modified the statement to say that perhaps there was but it was so subtle he could not tell what it was. The patient was usually continent at the time of discharge two weeks after operation, but still occasionally voided in bed.

Case 4 SS, WF, 61. This patient was admitted March 20, 1947, because of intractable pain in the face and head. About three and one-half years before she had developed a painless swelling at the angle of the left mandible. A sarcoma was removed and deep X-ray therapy, as well as Radon implantation was carried out. For three years she had constant, severe pain involving the entire left side of the head. There was continuous complaint. The patient was worried. Codeine and empirin were taken in large doses. Lobotomy was performed on March 22, 1947. Her complaints of pain stopped instantly and no more medication was required. On the fourth day after operation the patient began to run an unexplained fever, the cause of which was difficult to track down because there was no complaint of pain. About two days later it was discovered that the patient had a diffusely tender and rigid abdomen. X-rays revealed the presence of free air beneath the diaphragm and the diagnosis of perforated peptic ulcer with peritonitis was made. Because of the hopeless nature of the patient's tumor, no surgical

therapy was instituted and she died on the tenth day after lobotomy. Autopsy confirmed a perforation of a chronic peptic ulcer and showed a fibrinopurulent peritonitis. In addition there was a fresh coronary thrombosis.

In view of the known excruciating character of the pain from an acute perforation of a peptic ulcer, it is interesting that this patient made no complaint and seemed not uncomfortable. Yet she reacted in perfectly normal fashion to palpation of or pressure on the abdomen, wincing when this was done and showing the characteristic rigidity of the abdominal wall.

Case 5 M W, CF, 26. This patient was first admitted to the hospital in 1936, when she had several radical sinus operations, with uneventful recovery. In January, 1947 she was readmitted with a history of frontal headaches for about one month. X-rays showed what appeared to be a huge osteoma involving the left frontal bone, the orbit, and the floor of the anterior fossa of the skull. In February, 1947, operative removal of the osteoma was attempted, but was not successful because of the size, the infiltration and vascularity of the tumor. Pathologically it proved to be a carcinoma, probably arising in the nasal sinuses. The patient continued to have severe pain in the face and the left frontal region. On September 5, 1947, a prefrontal lobotomy was done. The patient at this time was about seven months pregnant. At the time of discharge, on the fifth postoperative day, she was confused, disoriented and incontinent, but had no pain. When seen a week later she seemed very happy and the family felt she was getting along very well without medication. On September 20, 1947, this patient was delivered of a normal, premature infant without anesthesia or analgesia, following a labor of five hours. Her reactions to the labor pain were interesting in that they appeared to be those of a normal woman under the influence of sedation. In other words, when a pain came, she screamed and yelled in ordinary fashion, but as soon as the pain was over she relaxed in perfectly normal manner and had no fear or anticipation about the next pain.

In addition to these five cases which have been described in some detail, there have been six others, with carcinoma of the kidney, the neck, breast, cervix and colon, and one case with a combined long standing carcinoma of the nose with a more recent carcinoma of the prostate. One patient died of intracranial hemorrhage three days after operation, the others are either free of pain and receiving no medication, or have died of their cancer.

COMMENT

Examination of all these patients after operation makes it clear that prefrontal lobotomy alters the individual's reactions to pain without materially changing his ability to feel pain. Ordinary tests of pain perception by pin-prick or other methods reveal that the

patient's perception of pain is unchanged. When stuck with a pin such a patient will wince, but it may be difficult to make him angry by repeatedly sticking him. The patient who died of peritonitis following perforation of a peptic ulcer showed a normal amount of tenderness and rigidity in the abdomen, yet never once complained of abdominal pain.



FIG 1 Showing so-called ideal plane of section from coronal suture to sphenoidal ridge

From the study of our cases it becomes clear that the real indications for lobotomy in cases of intractable pain are based in large part upon the emotional component, i.e., the "reaction" of the individual to his pain with anxiety, discouragement and apprehension. Prefrontal lobotomy will eliminate this affective component in a striking manner. In brief, it seems that when the fear of pain is abolished, the perception of pain itself is not intolerable. In the words of Freeman and Watts, "Pain may be present, but when divorced from its implication—

insecurity, disability, guilt, death—it then becomes bearable and may be accepted with fortitude'' The fear of impending death and the hopelessness of the situation are removed to the extent that the patient's prognosis can be discussed with him quite openly and truthfully He is no longer afraid to die

Prefrontal lobotomy relieves pain by interrupting the pathways at a psychologic rather than a physiologic level There is no specificity



FIG 2 The section in this case was considerably anterior to the ideal, yet the patient was relieved of pain

for pain, relief depends upon alteration of affective aspects of the personality For this reason partial lobotomies or attempted differential lobotomies have thus far been unsuccessful All four quadrants of both frontal lobes must be severed There is much to learn, however, about proper level of section* Theoretically, a proper lobotomy involves severance of the white matter from the coronal suture to the sphenoidal ridge Whether this extensive procedure is always neces-

sary in patients with intractable pain remains to be seen. It appears that the larger the section, the greater the alteration of personality.

The use of prefrontal lobotomy in patients with intractable pain has opened up a large field for study by psychiatric and psychologic methods. Research on the influence of the frontal lobes on personality and intelligence now may be attacked in much more direct fashion. This was not very often possible in the cases of lobotomy in psychotics, because proper pre-operative standards frequently could not be established. The cases presented in this series were not suitable for such a study because in every case post-operative life expectancy was so short that, although the patient was relieved of pain, his psychic functions did not often have time to reach their ultimate base line. Lobotomies done on patients with phantom limb pain, tabes dorsalis, or other non-lethal painful states will supply much more opportunity for study.

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- * Figures 1 and 2 indicate the possibility of variation in the level of section. In Figure 1 the so-called "ideal" plane of section is accomplished, the cut being made from the coronal suture to the sphenoidal ridge. In Figure 2 the section is a considerable distance anterior to this. In both instances the lobotomy brought relief of pain. In the first there was a major degree of personality alteration in addition. In the second, although pain relief was complete, there was no detectable gross alteration of personality. This comparison may indicate the direction of future work.

THE CARDIAC MECHANISM DURING ANESTHESIA AND OPERATION IN PATIENTS WITH CONGENITAL HEART DISEASE AND CYANOSIS

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The purpose of the present study is twofold (1) to determine the mechanism, significance, and management of the cardiac arrhythmias occurring during anesthesia and operation in children with congenital heart disease and cyanosis, and (2) to determine the preterminal cardiac mechanism in the children who die during this period. The importance of the latter problem, and what if anything might be done about it, is established by the relatively high incidence of death during operation, exceeded only by cerebral thrombosis as a single cause of death in the operative series.

The need for more adequate information concerning the cardiac mechanism during anesthesia and operation is apparent upon reviewing the available literature. Widely varying opinions have been expressed, with the result that a serious degree of confusion still exists about methods for the prevention and management of important cardiac arrhythmias. Certain shortcomings, more or less common to the majority of the studies published, are probably responsible for these variations of opinion, and include the following: (1) limited numbers of cases, (2) insufficient control of numerous and important variable factors, (3) dependence on the results of animal experiments without any necessarily direct comparison with results in human subjects, and (4) frequent lack of direct electrocardiographic evidence to support proposed hypotheses. Although it is not claimed that all of these criticisms have been avoided in the present investigation, the unusual opportunity for studying the heart's action during anesthesia and operation, especially under conditions of anoxia, has been productive of results which have appeared to be worthy of publication.

MATERIALS AND METHODS

Patients selected for the study included 175 consecutive children with congenital heart disease and cyanosis submitted to the Blalock-

Taussig operation (8) for the correction of the abnormal circulatory dynamics of pulmonic stenosis with intracardiac right-to-left shunt. Three standard extremity leads were recorded almost continuously using a direct-writing electrocardiograph. Preoperative medication consisted of morphine (1 mg per 5 kg body weight) and atropine (0.05 mg per 5 kg body weight) given hypodermically. Induction was accomplished with cyclopropane and oxygen, and this mixture with the addition of ether in small amounts, given intratracheally, was continued throughout operation except for changing in certain individuals to just ether and oxygen or occasionally to oxygen alone (12). Similar electrocardiographic records were made in a control group including ligation of a patent ductus arteriosus, 15 cases, surgical correction of coarctation of the aorta, 3 cases, and resection of the pericardium for constrictive pericarditis, 2 cases.

RESULTS

The general incidence of disturbances of the cardiac mechanism during anesthesia and operation in this series of children with congenital heart disease and cyanosis is approximately the same as in the non-cyanotic group and in other reported series of various types of operations in which cyclopropane was the chief anesthetic agent (16). This observation would appear to confirm the statement that the anesthetic agent is of greater importance than the type of operative procedure in the production of cardiac irregularities. The types of disturbance in the cardiac mechanism are also generally similar to those reported in other studies (10, 16, 20, 30, 31, 32), and may be classified as follows:

- 1 Simple variations in rate
 - Sinus tachycardia
 - Sinus bradycardia
- 2 Ectopic arrhythmias
 - A Displacement of the normal pacemaker
 - Atrio-ventricular nodal rhythm
 - B. Premature contractions and paroxysmal tachycardia
 - Auricular
 - Nodal
 - Ventricular

- 3 Abnormalities of the form of the ventricular deflections
 - Minor aberration of QRS
 - Bundle branch block
 - ST segment deviation
 - T wave inversion
- 4 Disturbances of conduction
 - PR interval
 - QT interval
- 5 Terminal mechanisms
 - Cardiac arrest
 - Complete A-V dissociation
 - Idioventricular rhythm
 - Ventricular fibrillation

For further details reference may be made to Tables I and II

Variations in heart rate with normal sinus mechanism are seldom of any significance. Sinus tachycardia was found to occur during all stages of operation and could be attributed in many instances to the effect of atropine given either as a routine preoperative medication or during the course of anesthesia and operation. Sinus bradycardia, on the contrary, was most frequently observed under circumstances apparently favoring vagal stimulation as evidenced by the fact that it was abolished in most cases by the administration of atropine (Fig 1)

Ectopic arrhythmias are of two general types depending on their mode of origin. They may result from (1) depression of the rhythmicity of the normal cardiac pacemaker (sino-auricular node) with supercedence of a center of otherwise lesser degree of rhythmicity, or (2) increased irritability of any portion of the heart, the new focus gaining control over the normal cardiac mechanism by virtue of its abnormal rhythmicity or by circus movement of the ectopic impulse. Arrhythmias of the first sort were most common, occurring in more than half of the children. In all instances this took the form of some type of nodal rhythm which maintained temporary control of both ventricles and auricles, the latter by retrograde conduction, or of the ventricles alone, the auricles responding to a slow sinus rhythm. Various types of nodal rhythm observed included the following: (1) nodal rhythm with absence of visible P waves which are presumably superimposed in QRS (Fig 1A and 2A), 24.7%, (2) classical AV nodal

TABLE I

	CYANOTIC	TERMINAL	NON CYANOTIC	TERMINAL
Number of cases	165	10	19	1
Incomplete	7		0	
Total	158	10	19	1
Regular throughout	35 (22 1%)	0	5 (26 3%)	0
Displacement of the normal pacemaker	88 (55 7%)	6 (60%)	6 (31 5%)	1
Abnormal rate	44 (27 8%)	10 (100%)	7 (37%)	
Sinus tachycardia	27 (17 1%)	9 (90%)	6 (31 5%)	1
Sinus bradycardia	17 (10 7%)	10 (100%)	2 (10 5%)	0
Premature contractions	14 (8 9%)	5 (50%)	4 (21%)	0
Auricular	1 (0 63%)	0	0	
Ventricular	12 (7 6%)	3 (30%)	4 (21%)	
AV nodal	1 (0 63%)	2 (20%)	0	
Aberration of QRS	14 (8 9%)	4 (40%)	3 (15 8%)	0
Bundle branch block	0	2 (20%)	0	1
Paroxysmal tachycardia	7 (4 4%)	7 (70%)	1 (0 05%)	
Auricular	7 (4 4%)	2 (20%)	0	
Ventricular	0	3 (30%)	0	1
ST segment deviation	7 (4 4%)	3 (30%)	0	0
T wave inversion	2 (1 26%)	0	0	0
Prolongation of PR interval	1 (0 63%)	0	0	0
Prolongation of QT interval	1 (0 63%)	0	0	
Sinus arrest	1 (0 63%)	9 (90%)	0	1
AV dissociation	0	9 (90%)	0	0
Terminal asystole	0	7 (70%)	0	1
Ventricular fibrillation	0	1 (10%)	0	0

rhythm with inverted P waves either preceding QRS with a short PR interval or immediately following QRS (Fig 2B and C, 6C, 7B), 19 0%, (3) AV nodal rhythm with interference dissociation (Fig 7C and D), 12%, (4) coronary nodal rhythm with upright P waves and

short PR interval (Fig 1G and 2D), 7.6%, and (5) high nodal rhythm with inverted P waves preceding QRS with a normal or only slightly shortened PR interval (Fig 6C), 5.7%. Although these various types of disturbance appear superficially to represent a single basic mechanism, it seems likely from a more detailed analysis that they

TABLE II

	TOTAL NO	ANESTHESIA	MEDIASTINAL EXPLORATION	ANASTOMOSIS	CLOSING
Displacement of normal pacemaker	88	36	48	49	29
Tachycardia	27	3 (4)	2 (4)	8 (4)	1 (4)
Bradycardia	17	8	9	0	0
Premature Contraction					
Auricular	1	0	0	1	0
Ventricular	12	4	6	2	0
Nodal	1	0	1	0	0
Aberration of QRS	14	2 (7)	3 (7)	2 (7)	0 (7)
Paroxysmal tachycardia					
Auricular	7	1	1	0	5
Ventricular	0	0	0	0	0
ST segment deviation	7	0	0	7	0
T wave inversion	2	2	(2)	(2)	(2)
Prolongation of PR interval	1	0	0	1	0
Prolongation of QT interval	1	0	0	1	0

result from the interaction of at least two different mechanisms. Thus, it was observed that the ventricular rate during AV nodal rhythm exceeded that during the preceding normal sinus rhythm in 33 instances (25.5%), remained the same in 67 (51.9%), and was slower in 29 (22.6%). The latter group is probably the only one that represents a true passive nodal rhythm, with depression of the normal pace-

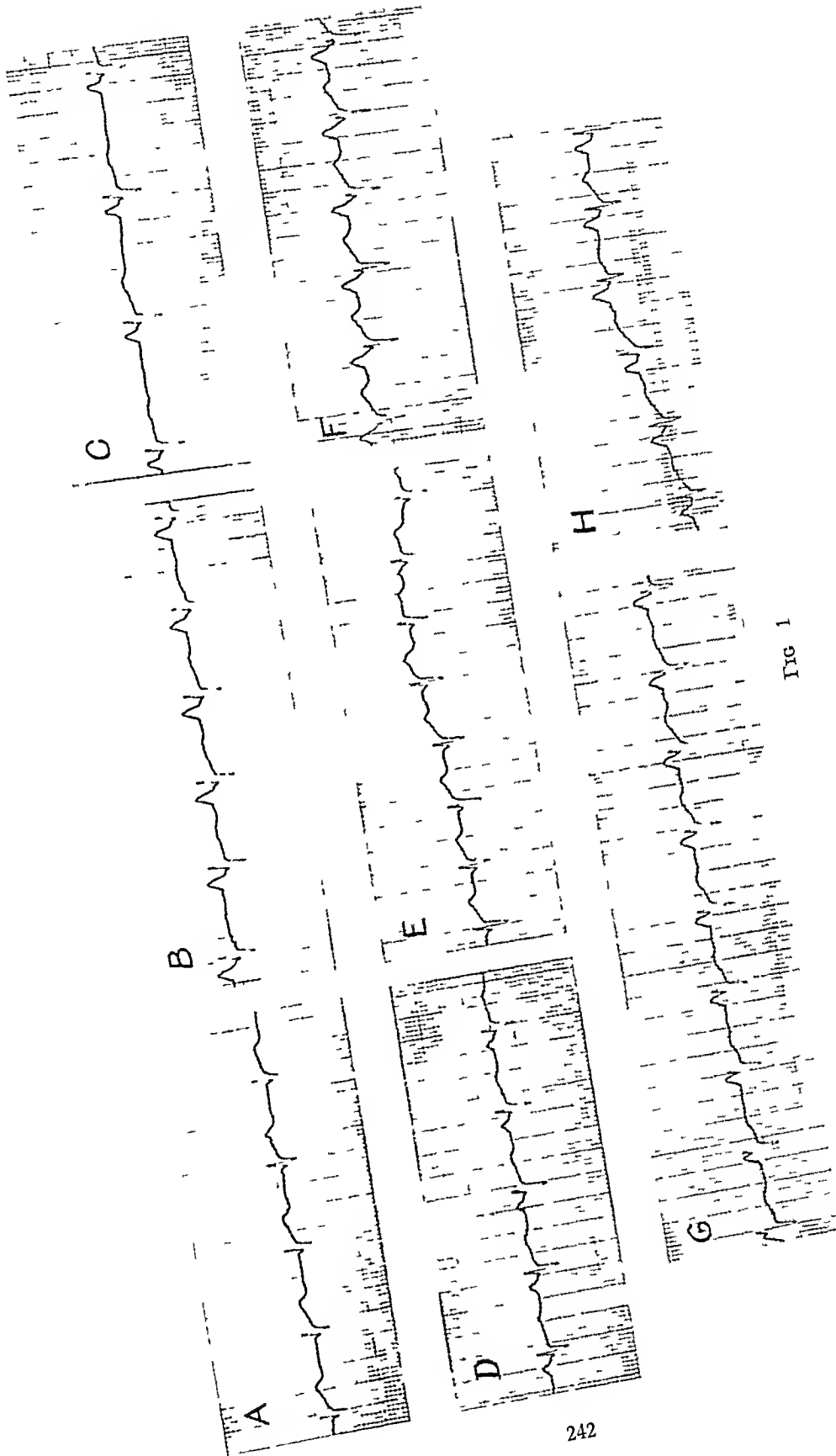


Fig. 1

maker (SA node) and supercedence of a center of lower rhythmicity (AV node) The first group, with a faster ventricular rate by virtue of an abnormally increased degree of rhythmicity of the AV node (Fig 1E), appears to belong to what may more properly be termed an active ectopic nodal tachycardia The intermediate group probably comprises both mechanisms, that is, abnormal depression of the rhythmicity of the SA node together with abnormal acceleration of that of the AV node In support of the foregoing concept are data derived from an analysis of the response of the heart controlled by the AV node to the administration of atropine More than half of the cases (46, 52 3%) reverted spontaneously to normal sinus rhythm, 19 (21 6%) reverted with atropine (Fig 2A and C), 8 (9 1%) were basically unaffected by atropine, and 15 (17%) could not be classified In all instances of slow nodal rhythm, reversion to a normal sinus mechanism was accomplished by the administration of atropine, indicating that the stimulus for depressing the normal rhythmicity of the sino-auricular node was probably mediated by way of the vagus nerves Those that failed to respond to atropine, on the contrary, were all instances of fast nodal rhythm or nodal tachycardia, indicating that the primary mechanism in this group was something other than vagal depression of the normal cardiac pacemaker That vagal stimulation might be exerting at least a minor influence in this group, however, was evidenced by the fact that in several instances the ventricular rate, though still controlled by the AV node, became more rapid after the administra-

FIG 1 R K, AGE 22 YEARS, TETRALOGY OF FALLOT
AV NODAL RHYTHM LEAD 2

- A Nodal rhythm with absent P waves, rate 80, during anesthesia
- B Spontaneous normal sinus rhythm, rate 84 (without atropine)
- C Sinus bradycardia, rate 55, mediastinal exploration
- D Normal sinus rhythm after atropine, rate 84, difference in the form of the P waves from that of earlier and later portions of the record, mediastinal exploration
- E Fast nodal rhythm with P superimposed in QRS, rate 106, during anastomosis
- F Normal sinus rhythm, rate 88
- G Nodal rhythm with dissociation, variation in the relation of P to QRS, during anastomosis
- H Normal sinus rhythm, rate 94, closing chest

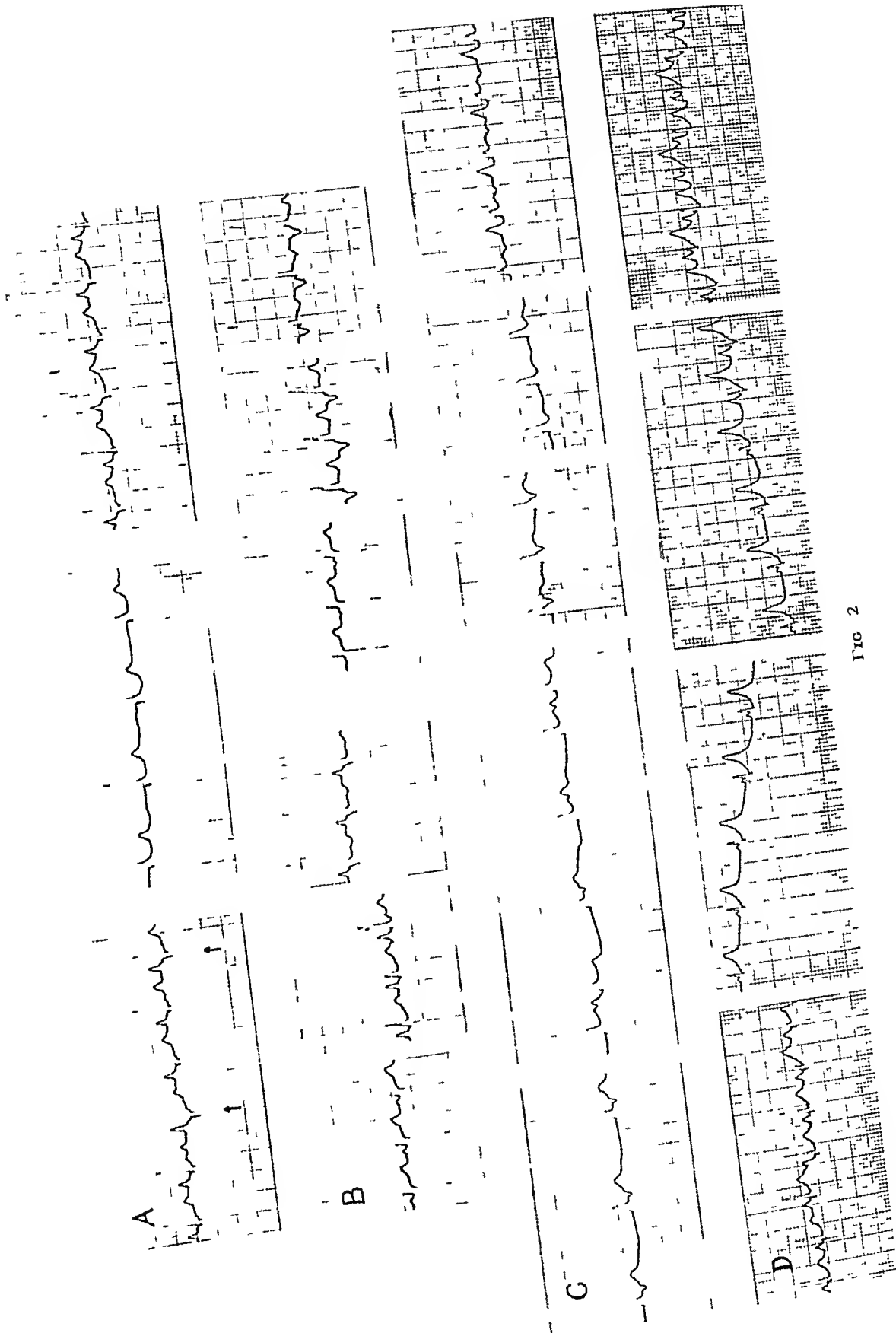


FIG. 2

tion of atropine (Fig 1E, 2C) Nodal rhythm of the various types listed occurred with nearly equal frequency during anesthesia and throughout all phases of operation and was not related to any specific operative procedure It may therefore be assumed that, while direct vagal stimulation as in mediastinal exploration and manipulation of the great vessels may be responsible for the occurrence of such arrhythmias, they may also result from action of the anesthetic agent mediated by way of the vagus nerves as well as by direct action on the myocardium and the specialized tissues of the heart The effect of pre-operative medication also must be considered, morphine tending to exaggerate the arrhythmias in part perhaps by vagal stimulation, and atropine failing to protect the heart against such irregularities in the amounts usually given (11) It may be stated that morphine is claimed to have a protective effect against ectopic arrhythmias such as cyclopropane-epinephrine tachycardia (3)

Arrhythmias due to increased cardiac irritability, including premature systoles and paroxysmal tachycardia, occurred in a total of 21

FIG 2 FOUR CASES DEMONSTRATING VARIOUS TYPES OF NODAL RHYTHM

A T S, age 6 years, tetralogy of Fallot Lead 3

Normal sinus rhythm, rate 110, spontaneous variation in form of QRS

Nodal bradycardia with absent P waves, rate 75, mediastinal exploration

Normal sinus rhythm after atropine

B A M, age 5½ years, tetralogy of Fallot Leads 1, 2, and 3

Normal sinus rhythm, rate 120

Nodal rhythm with inverted P wave preceding QRS by slightly shortened PR interval, opening of anastomosis

C R K, age 15 years, tetralogy of Fallot Lead 2

Inverted P wave following QRS, rate 65, pre operative

AV nodal rhythm with apparently "premature" systoles arising from normal pacemaker in SA node

Nodal tachycardia after atropine, rate 115 P waves at first inverted and following QRS, later absent

Normal sinus rhythm, rate 106

D M C, age 2 years, tetralogy of Fallot Lead 3

Normal sinus rhythm, rate 150

Nodal rhythm with upright P wave preceding QRS with short PR interval, rate 96, during anastomosis

Nodal rhythm, emergence of P wave from QRS

Normal sinus rhythm occurring spontaneously, rate 150

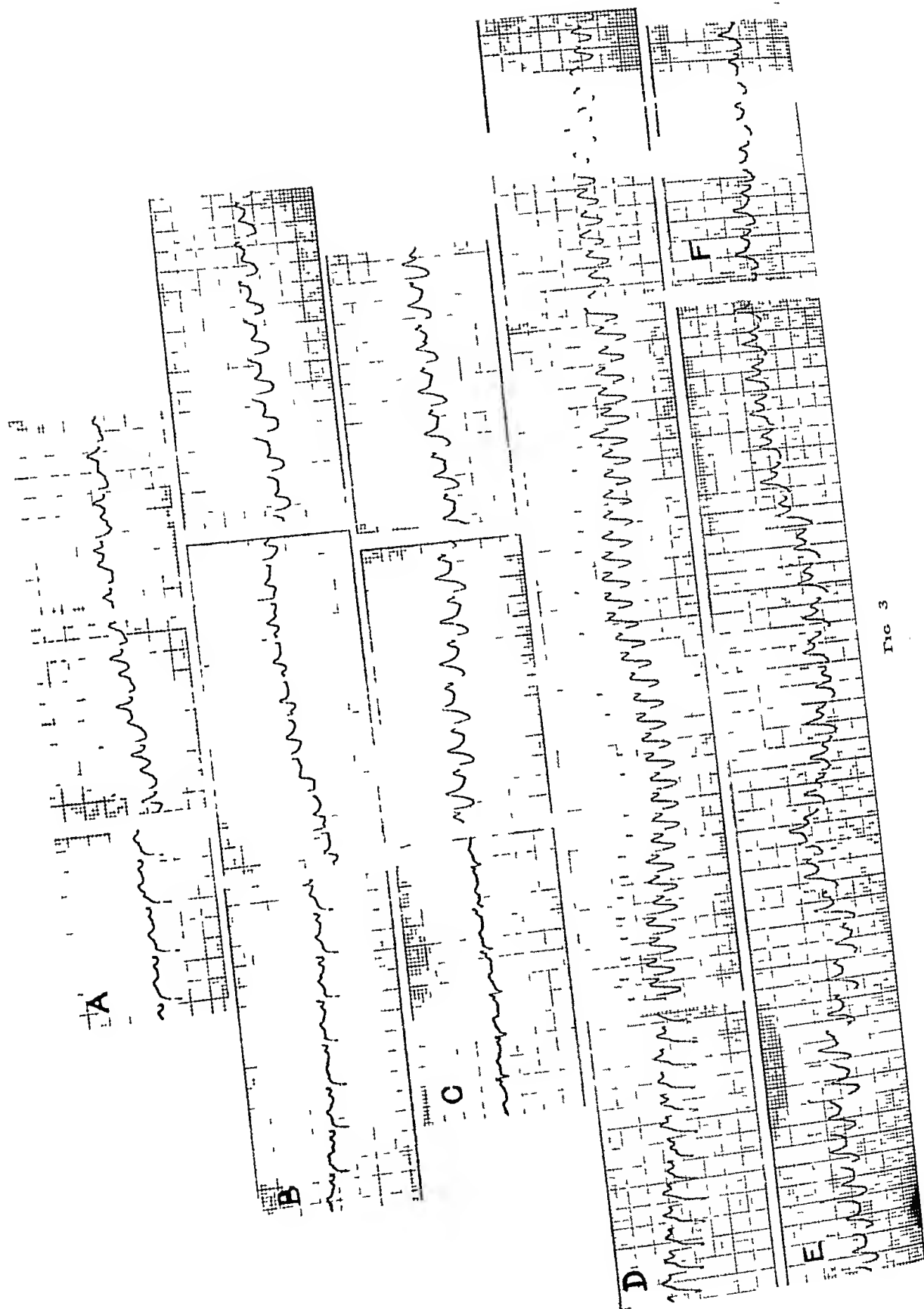


FIG. 3

patients (13.3%) Of the premature contractions, 12 of the 14 were ventricular, most occurred early in operation, and at least half were definitely due to cyclopropane, disappearing rather promptly after changing the anesthetic mixture to ether and oxygen (Fig 4) Auricular premature systoles occurring in one case were due to direct stimulation of the right auricle, and nodal premature systoles in one were apparently the direct result of manipulation of the vagus nerve Paroxysmal tachycardia, except when arising in the AV junctional tissues, was of auricular origin in every case of the surviving group The majority of instances (5 of 7, 71.5%) occurred at the end of operation during chest closure (Fig 3), one during anesthesia before the chest was opened, and one during mediastinal exploration and anastomosis The cause of this disturbance of the cardiac mechanism is not clear, although it is known, and was observed in this series, that stimulation of the vagus nerve tends to abolish the arrhythmia whereas paralysis of the vagus by atropine tends to perpetuate it

Another type of disturbance of the cardiac mechanism, which can be detected only by electrocardiography, is the group of abnormalities of the form of the ventricular deflections In this group are included minor aberration of the form of QRS 14 (8.9%), ST segment deviation 7 (4.4%), and T wave inversion 2 (1.3%) Minor variations in the form of QRS, of academic interest but apparently of no great clinical significance, ranged from slight changes in the amplitude of the ventricular deflections (Fig 3B) to gross reversal of the direction of

FIG 3 M W, AGE 7 YEARS, TETRALOGY OF FALLOT

Paroxysmal supraventricular tachycardia with reversion to normal after intravenous digitalis

A Leads 1, 2, and 3, normal sinus rhythm, rate 125, pre operative

B Leads 1, 2, and 3, spontaneous aberration of QRS (lead 1), nodal rhythm with interference dissociation (lead 2), nodal rhythm and ST segment depression (lead 3), during anastomosis

C Leads 1, 2, and 3, nodal rhythm with upright P waves and short PR interval, rate 150, depression of ST segments leads 2 and 3, completion of anastomosis

D Leads 1, 2, and 3, paroxysmal supraventricular tachycardia, rate 300, chest closed

E Lead 2, gradual emergence of P wave to normal sinus rhythm after intravenous digitalis (Lanatoside C)

F Lead 2, normal sinus rhythm, rate 130

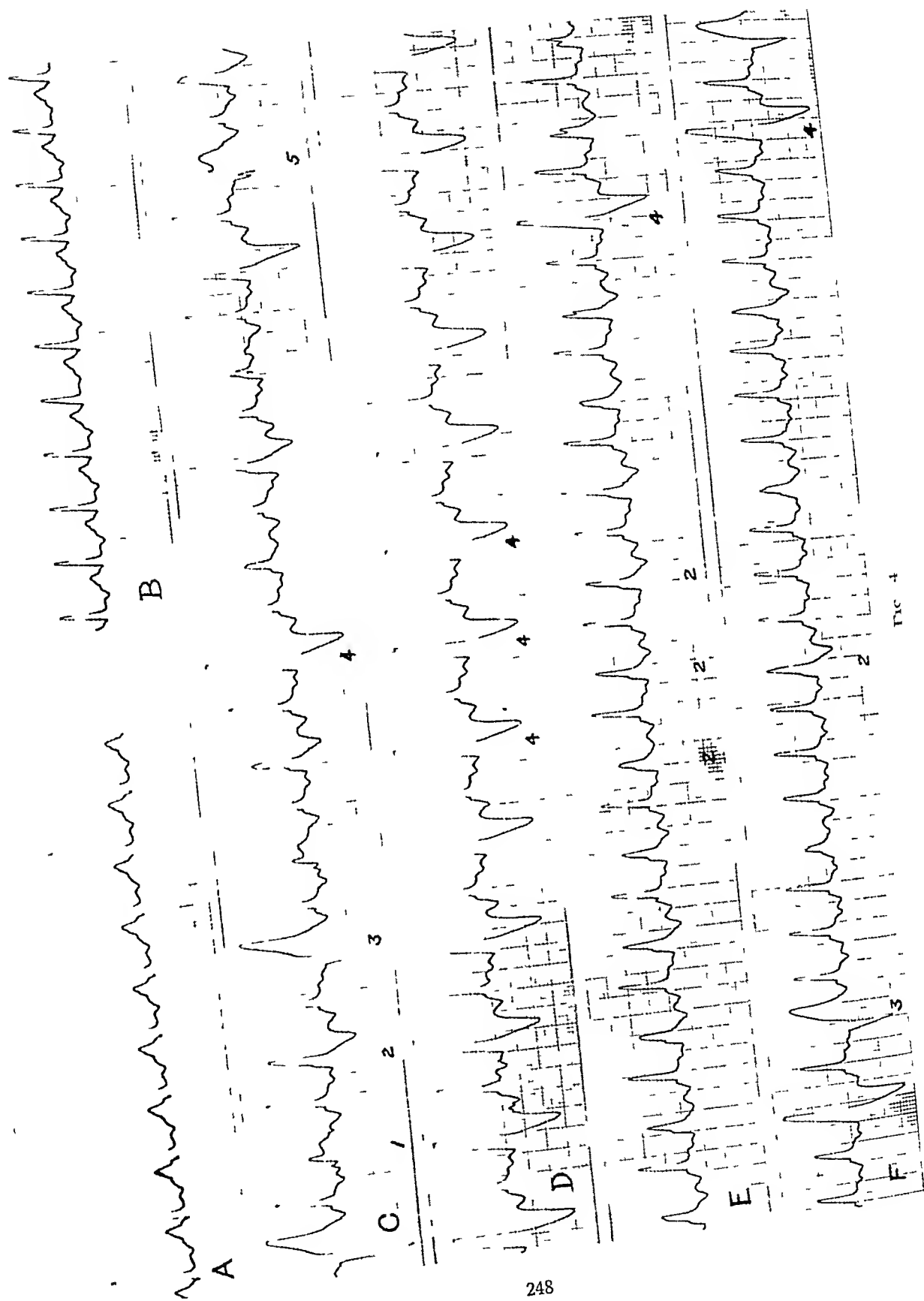


FIG. 4

QRS and consequently of the mean electrical axis of the heart (35). These changes occurred throughout all stages of operation and could not be associated with any particular event. They appeared at times to be cyclic as though varying with the phase of respiration and the position of the heart in the thorax, at other times, however, they appeared, disappeared, and reappeared without any apparent explanation. It is assumed that such variations in the form of QRS probably represent relatively minor changes of intraventricular conduction. Bundle branch block was observed only in the terminal cases, but was simulated in five additional instances by the superimposition of P waves on QRS during AV nodal rhythm (Fig 5). ST segment and T wave changes occurred predominantly under circumstances indicating the probability of myocardial anoxia as a causative factor, once during paroxysmal tachycardia (Fig 3), and in the remaining instances becoming most marked with the chest open and lung collapsed (Fig 6) or just before release of the clamps after completion of the anastomosis.

Disturbances of conduction were infrequent, and their significance uncertain. It may be stated simply that such factors as autonomic imbalance and anoxemia are known to cause such changes as those observed.

In the terminal group were included seven children who died during anesthesia or operation, two children who experienced several episodes of cardiac asystole on the operating table and who died within 24 hours of cerebral thrombosis probably occurring during asystole, and one additional child whose operation was postponed after surviving what was thought to be a series of preterminal arrhythmias during anes-

FIG 4 W. G., AGE 16 YEARS, TETRALOGY OF FALLOT

Ventricular premature systoles resulting from effect of cyclopropane, regular rhythm after changing to ether and oxygen. Lead 3

A Normal sinus rhythm, pre-operative

B Normal sinus rhythm, postoperative

C Ventricular premature systoles from multiple (5) foci, mediastinal dissection

D and E Bigeminal rhythm with ectopic focus at first in one and then in the other ventricle

F Gradual subsidence of active arrhythmia after stopping cyclopropane, inverted P waves preceding QRS with normal PR interval

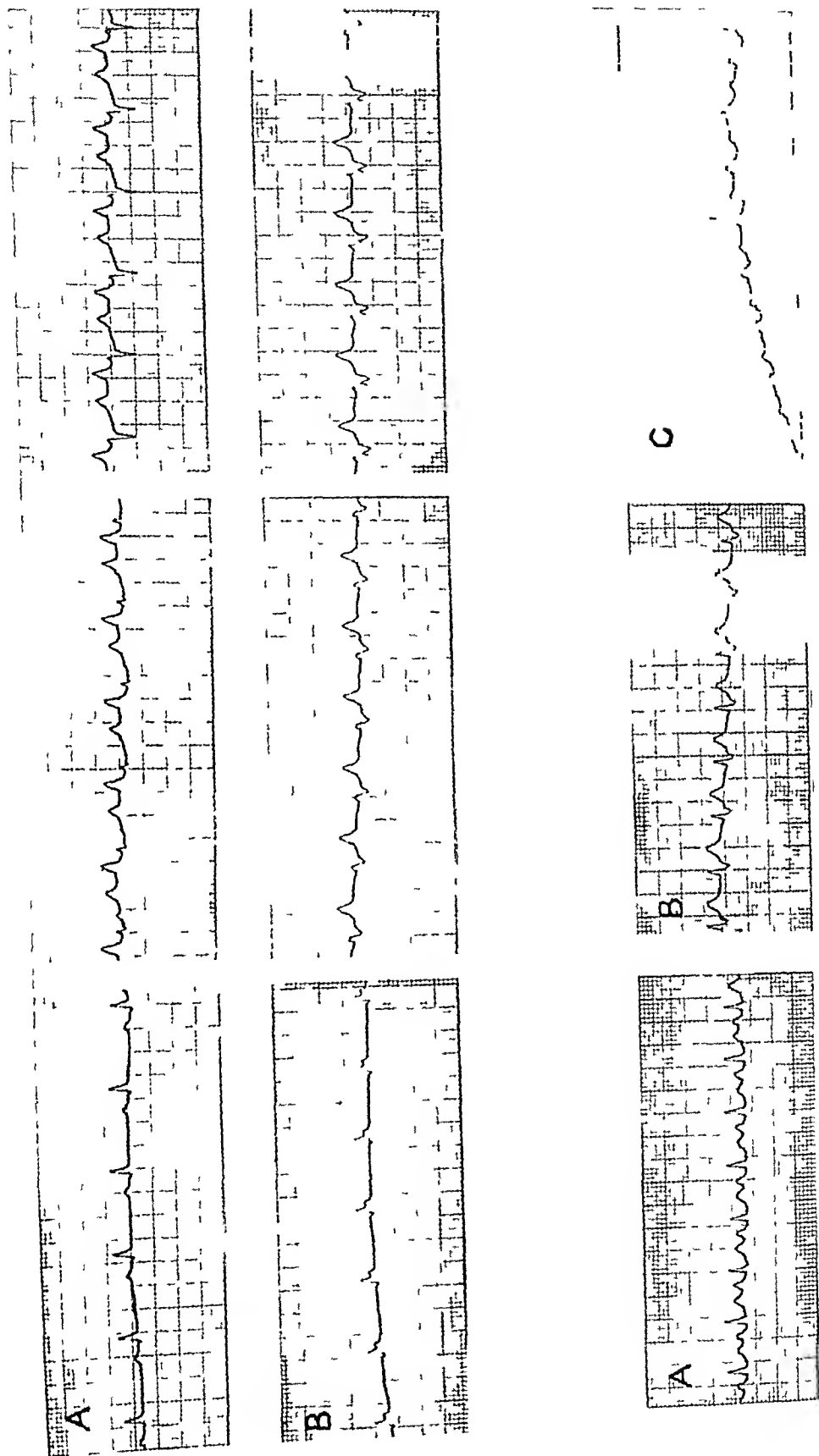


FIG 5

thesia Though this group is relatively small, certain differences of probable significance were observed between them and the larger control series

The terminal cardiac mechanism in each of the seven cases was cardiac asystole (Fig 8 and 9) It is interesting and probably of some importance that ventricular fibrillation occurred only once, and in this case long after death had already resulted from prolonged asystole (Fig 9) Despite the fact that isolated cases of survival have been reported following long periods of cardiac arrest (1, 5), it was at first believed that the earliest onset of hypodynamic heart action marked the beginning of an irreversible terminal mechanism The occurrence, however, of three patients surviving this period of asystole, while in no way decreasing the ultimate importance of such an event in this type of case, led to the newer belief that something might be done about it especially if any warning of impending danger might be foreseen The records were carefully analyzed with this in mind, and certain observations of probable significance were noted

In every case except one, sinus tachycardia with a rate of 150-200 preceded the onset of any other abnormality of the cardiac mechanism While this incidence is significantly higher than in the control group, there is nothing sufficiently distinctive about it to forewarn of anything more serious to follow Of much greater significance was marked bradycardia, either sinus or AV nodal, occurring in every case prior to terminal asystole This form of bradycardia differed in several important respects from that occurring in the control series The rate in the terminal group ranged from 23-50 per minute, while in the control group it was never less than 55 and in most cases was considerably faster In five cases of the terminal group the bradycardia either

FIG 5 TWO CASES DEMONSTRATING SIMULATION OF BUNDLE BRANCH BLOCK, THE RESULT OF SUPERIMPOSITION OF P WAVES ON QRS DURING AV NODAL RHYTHM

Upper two records—M C, age 18 years, pulmonary stenosis and possible single ventricle Left axis deviation in the extremity leads (and left ventricular hypertrophy in the precordial leads) A Leads 1, 2, and 3, normal sinus rhythm, rate 88, pre operative B AV nodal rhythm, during anastomosis

Lower record—W B, age 11 years, probable tetralogy of Fallot A Lead 2, normal sinus rhythm, rate 125, pre operative B Lead 2, AV nodal rhythm, rate 125, during anastomosis C Lead 3, emergence of P wave from QRS

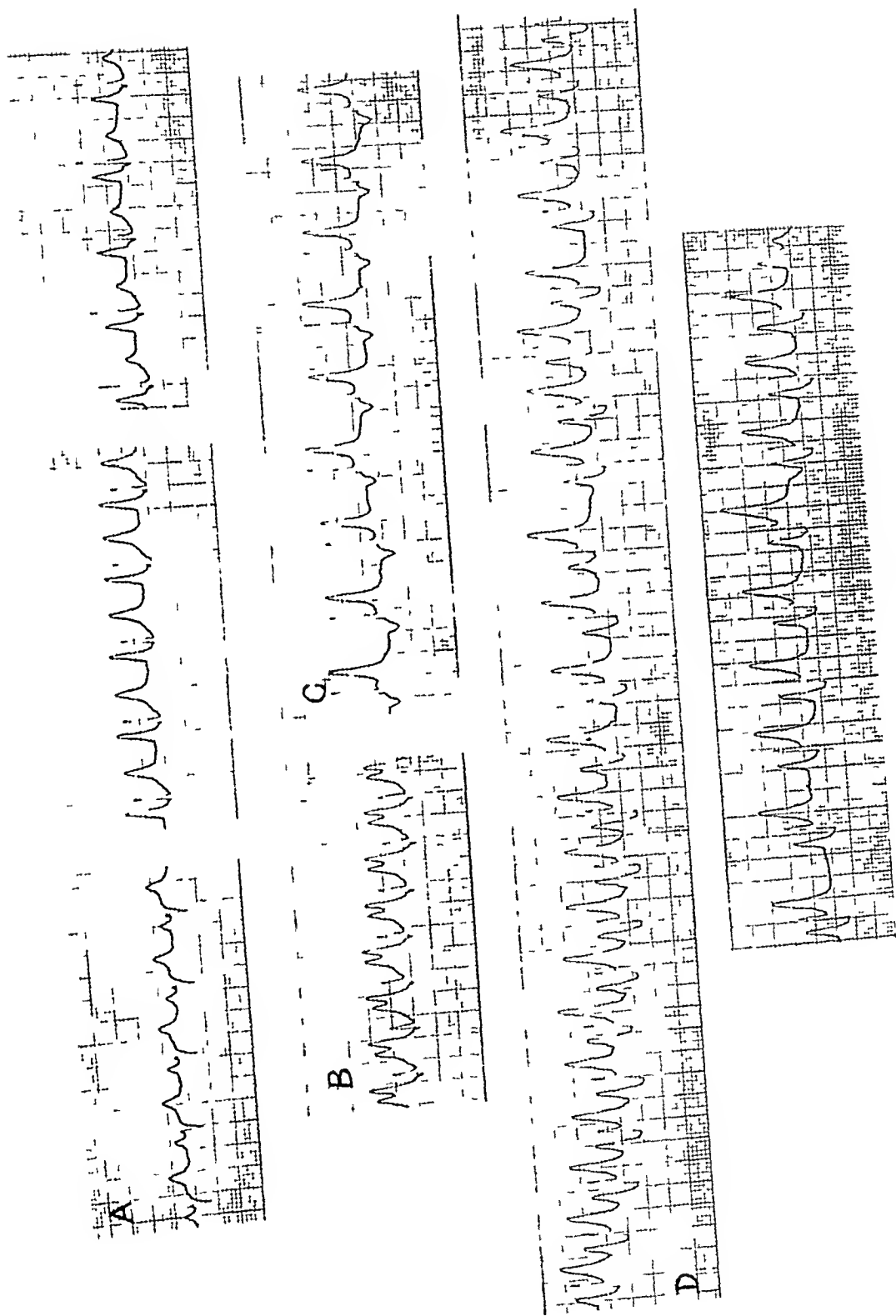


FIG 6

occurred despite the administration of atropine or failed to revert to normal after atropine. In the control group, on the contrary, those with bradycardia invariably responded to the administration of atropine with an increase in heart rate, the only ones failing to respond already having a ventricular rate faster than during the preceding sinus rhythm. Of the nine children who died, only one-third (3 cases) proceeded to terminal asystole during the first episode of bradycardia (Fig 8) while two-thirds (6 cases) had at least one and sometimes several episodes of bradycardia prior to the terminal event. It therefore seems evident that bradycardia, either sinus or nodal, with a rate of less than 50 per minute, and with failure to respond to the administration of atropine, constitutes a specific warning of impending terminal asystole. Other abnormalities of the cardiac mechanism were observed in the terminal group with sufficient frequency to be considered important evidence of cardiac damage and the accompanying danger of preterminal arrhythmias. These included complete bundle branch block in two cases, ventricular tachycardia in three cases, important ST segment deviation in three cases, complete AV dissociation in nine cases including two patients with auricular rhythm but absence of ventricular response, and sinus arrest in nine cases. The two latter abnormalities only occurred terminally and therefore could not be utilized as a danger signal in time to make use of prophylactic or therapeutic measures. Complete AV dissociation never developed during operation in those who survived but was present preoperatively in four children, three of whom survived operation and one of whom died after a clamp was placed on what was later discovered to be a single pulmonary artery.

From the data thus far available one can only speculate as to the probable mechanism of terminal arrhythmias occurring during anesthesia and operation in children with congenital heart disease and

FIG 6 C B AGE 5 YEARS, TETRALOGY OF FALLOT

(Operation discontinued before doing anastomosis because of poor clinical condition of patient and evidence of progressive cardiac damage due probably to anoxemia)

A Leads 1, 2, and 3, normal sinus rhythm, rate 85, pre-operative

B Lead 2, sinus tachycardia, rate 150

C Lead 2, nodal bradycardia, rate 88, mediastinal exploration (inverted P preceding QRS with short PR interval)

D Lead 2, irregular sinus tachycardia (? sinus arrhythmia) after atropine, rate 83-135. Progressive ST segment elevation (normal after chest closure)

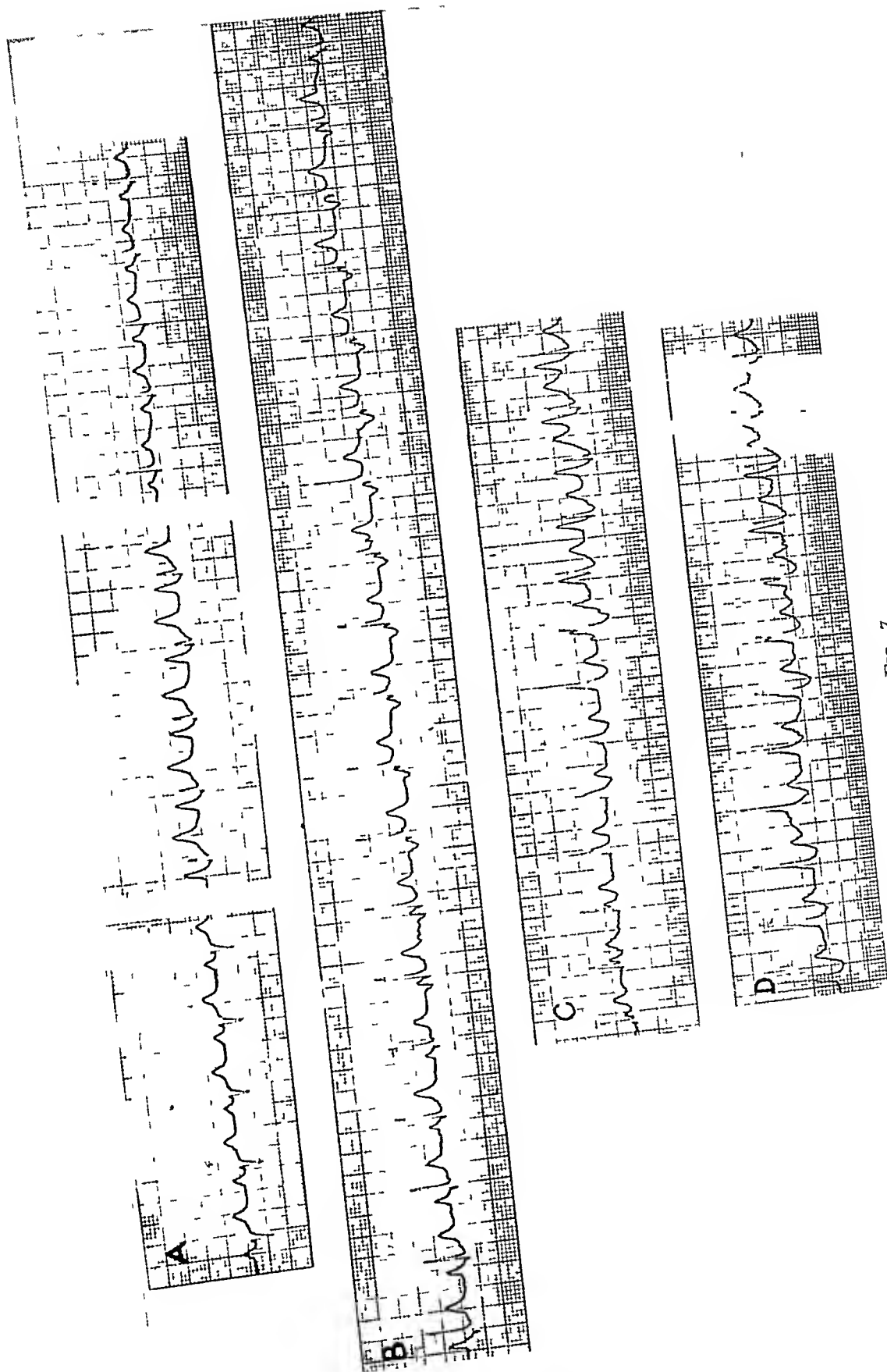


FIG 7

cyanosis. There is much to suggest that anoxemia is a factor of considerable importance. Thus, death was known to have followed almost immediately the clamping of a single pulmonary artery in two instances and the only large collateral vessel in a third. By analogy with these three cases it seems reasonable to postulate a similar mechanism of terminal anoxemia in cases in which the only pulmonary circulation, and therefore oxygenation of the blood, depends during operation (as well as at all other times) on relatively limited collateral circulation which becomes seriously compromised when the lung is collapsed during thoracotomy. This would include patients with complete pulmonary atresia, truncus arteriosus without pulmonary arteries (essentially inoperable), and tetralogy of Fallot with a single pulmonary artery, of which types there was a high proportion in the terminal group (11 of 18 autopsied cases, 61%) (Table III). In support of this concept is the low average arterial oxygen saturation in the terminal group and the occurrence of other electrocardiographic abnormalities known to depend upon myocardial anoxia, as in coronary insufficiency with or without myocardial infarction. On the other hand, it is difficult in terms of this concept to explain a number of contrary facts including the following: (1) death during anesthesia when the arterial oxygen saturation may be high initially or may rise above the pre-operative level after induction, (2) frequent survival of patients with supposed complete pulmonary atresia (clinical diagnosis), (3) death of children with uncomplicated tetralogy of Fallot, and particularly those with a relatively high arterial oxygen saturation. It seems evident that, although anoxemia may be an important factor, there must be other factors acting adversely during anesthesia and operation in this type of patient. Among these may be listed vagal stimulation (17, 25), other imbalance of the autonomic nervous system (4), direct effect of the anesthetic agent on a sensitized myocardium (19, 20, 22), decreased

FIG. 7 SAME PATIENT AS IN FIG. 6, SECOND ATTEMPT AT OPERATION WITH SUCCESSFUL COMPLETION OF ANASTOMOSIS

- A Leads 1, 2, and 3, normal sinus rhythm, rate 88, pre-operative
- B Lead 3, transition of P wave—upright, diphasic, inverted—representing progressive variation in position of pacemaker
- C and D Progressive alteration in relation of P to QRS, auricles responding to sinus impulses, ventricles responding to AV-nodal impulses, auricular rate slightly faster than ventricular, mediastinal exploration

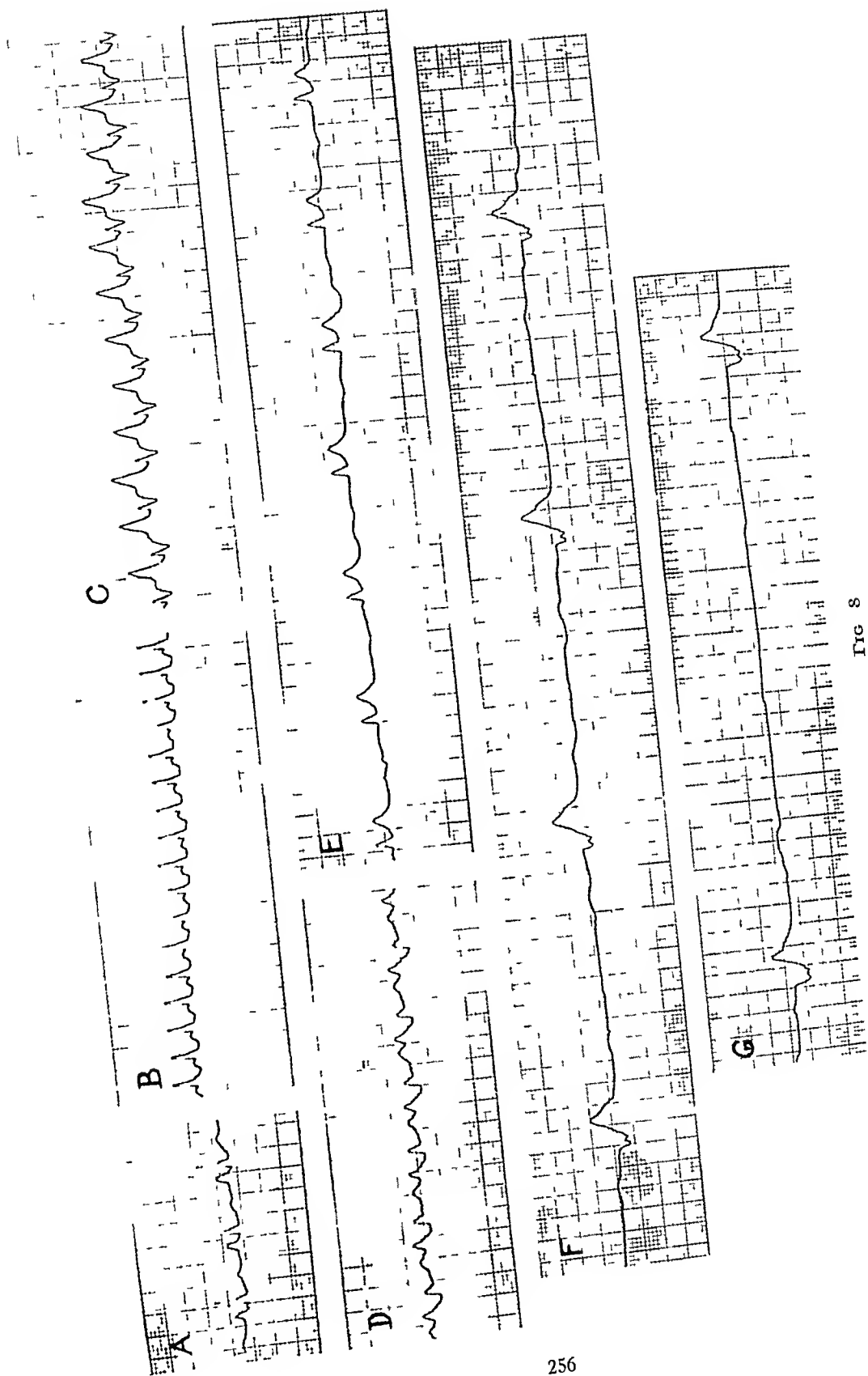


Fig 8

cardiac output with positive endotracheal pressure (15), adrenal cortical insufficiency, potassium inhibition, and perhaps others. From the viewpoint of specific treatment it is obvious that exact knowledge of all of the factors contributing to death during anesthesia and operation is of the greatest importance. From the foregoing observations it can be justifiable only to point out certain possibilities. Further investigation will still be necessary to clarify many of the problems encountered in a study of this sort.

DISCUSSION

Before discussing the management of the important cardiac arrhythmias occurring during anesthesia and operation it is necessary to consider the general value of the electrocardiogram in the detection of significant disturbances of the cardiac mechanism. In the first place, the electrocardiograph is an extremely sensitive instrument for recording variations in the heart's action. This is demonstrated by the fact that arrhythmias have been detected in approximately 80% of the cases electrocardiographically (16) but in only 6.5% by ordinary clinical means (30). This does not mean, of course, that all of these arrhythmias are of clinical significance, relatively few are actually of anything more than academic interest. On the other hand, the heart under direct observation was on a number of occasions seen to dilate and stop mechanically at the same time that the electrocardiogram continued briefly at least to show regular electrical impulses of normal rate and fairly normal configuration. It was also observed that regular electrical activity of some type continued long after clinical death of the patient and complete mechanical asystole. This seems reasonable since electrical systole need not necessarily bear any direct relation to mechanical systole, and it indicates further that an electrocardio-

FIG. 8. M. B., AGE 17 YEARS (PATIENT NUMBER 4, TABLE III)

Unexplained death before opening pleura. Lead 2.

A Normal sinus rhythm, rate 94, pre-operative.

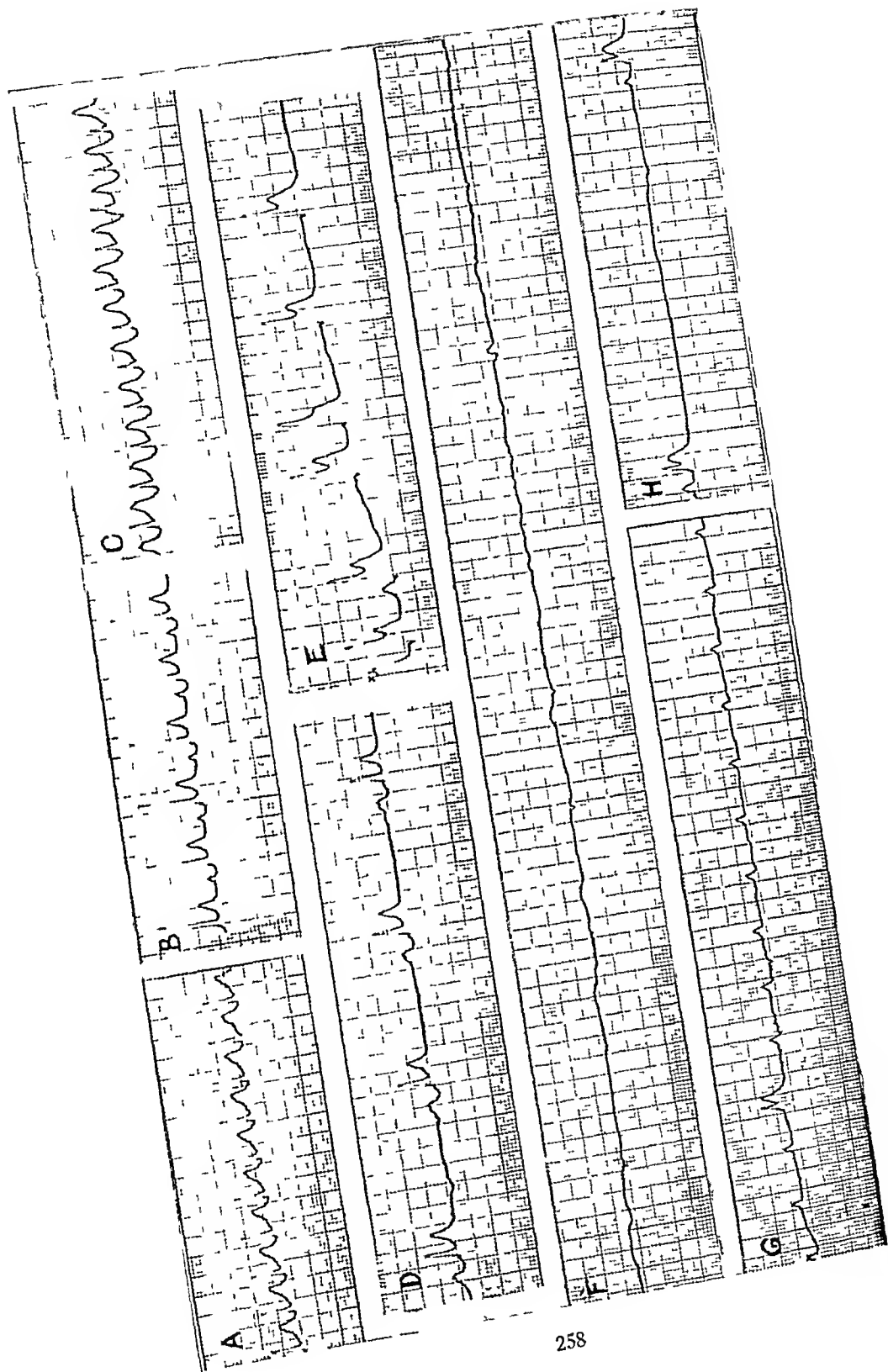
B Paroxysmal supraventricular tachycardia, rate 250, skin incision.

C Bundle branch block, or aberration of QRS by superimposed P wave.

D Normal sinus rhythm.

E Nodal bradycardia, rate 53, failure to respond to administration of atropine or adrenaline. Slight aberration of intraventricular conduction.

F and G Sinus arrest, idioventricular rhythm, terminal complete asystole.



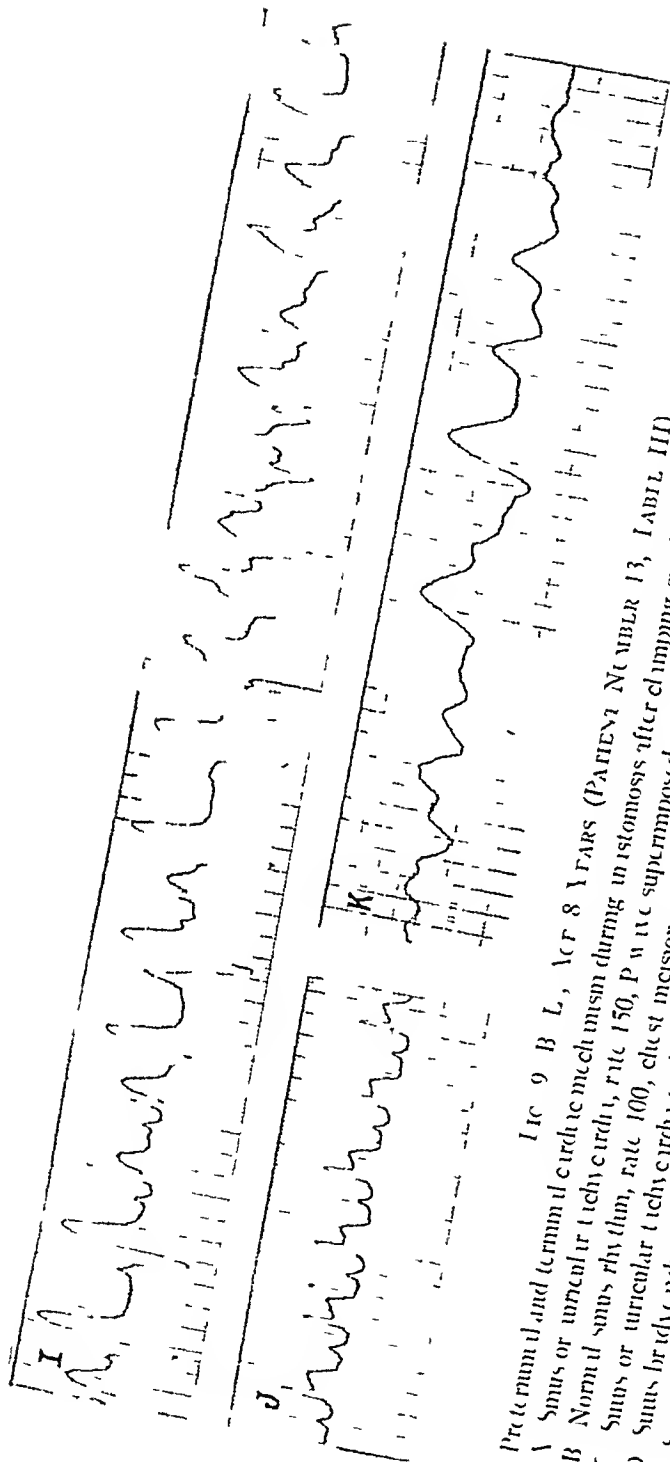


FIGURE 9. B. L., AGE 8 YEARS (PATIENT NUMBER 13, LABEL III)
 A Sinus bradycardia, rate 38, during anastomosis
 B Normal sinus rhythm, rate 100, chest incision
 C Sinus tachycardia, rate 150, P wave superimposed on preceding T, during anastomosis
 D Sinus bradycardia, rate 38, during anastomosis, after adrenaline and continue
 E Complete asystole and clinical death (cardiac massage)
 F Irregular rhythm without ventricular response, after calcium chloride
 G Slow sinus rhythm, rate 14 with SI segment elevation and short QT interval
 H Multiple ventricular premature systoles
 I Sinus rhythm with marked SI segment elevation
 J Ventricular fibrillation, approximately one hour after clinical death during asystole

TABLE III

TIME	AGE	DIAGNOSIS	O. SATU RATION	COMMENTS
			<i>per cent</i>	
Anesthesia				
1 M C (F)	22 yrs	—	66.8	Survived anesthesia
Opening chest				
2 N Q (M)	2½ yrs	Pulmonary atresia	42.0	Unexplained death be- fore chest opened
3 J P (F)	5 yrs	Pulmonary atresia	42.4	
4 M B (F)	17 yrs	Pulmonary stenosis, valvular, patent foramen ovale	72.2	
Mediastinal dissection				
5 B C (M)	4 yrs	—	36.5	Clamp on single pul- monary
6 M K (M)	7 yrs	Tetralogy	40.1	
7 G B (F)	2 yrs	Single ventricle	26.3	
8 I S (M)	7 yrs	Tetralogy, single pul- monary	65.2	
9 L W (M)	2½ yrs	Tetralogy	—	
During anastomosis				
10 C D (F)	8 yrs	Tetralogy	29.7	Clamp on single pul- monary
11 D K (M)	5 yrs	Tetralogy	41.9	
12 H G (F)	26 yrs	Pulmonary stenosis, tri- cuspid atresia	38.4	
13 B L (F)	8 yrs	Tetralogy, single pul- monary	34.7	
14 S S (F)	2 yrs	Truncus arteriosus, no pulmonaries	36.0	
15 S S (F)	3 yrs	Tetralogy	—	Clamp on single collat- eral
After completion of anastomosis				
16 D C (M)	8 yrs	—	46.4	Complete heart block acute dilatation
17 D B (M)	14 yrs	Pulmonary stenosis, valvular, single ven- tricle	64.2	
18 P N (M)	7 mo	Truncus arteriosus, no pulmonaries	13.4	Operation completed, cerebral thrombosis during asystole
19 K M (F)	2½ mo	Pulmonary atresia	17.5	
20 C F (F)	23 mo	Pulmonary atresia	7.5	
21 R D (F)	4½ mo	Pulmonary atresia	18.2	
22 B W (F)	4 yrs	Tetralogy	52.8	
23 I C (F)	3½ yrs	—	38.7	Operation completed, cerebral thrombosis during asystole
24 C H (M)	15 yrs	Tetralogy, anomalous venous return	63.0	Anomalous venous re- turn

graphic record, while of considerable value, does not completely replace direct observation in the detection of serious abnormalities of the heart's action during thoracic surgery. Somewhere between these

TABLE IV
Important Arrhythmias Pathogenesis and Management

	CAUSE	DANGER	TREATMENT
Ventricular premature systoles	Cyclopropane	Ventricular tachycardia and fibrillation	Stop cyclopropane, increase oxygen
Paroxysmal tachycardia	?	Congestive failure Peripheral collapse and thrombosis	Vagus stimulation (carotid sinus pressure) Intravenous digitalis
Nodal or sinus bradycardia	Vagal stimulation (direct or anesthetic)	? preterminal	Atropine
Nodal or sinus bradycardia below 50, no response to atropine, and other evidence of anoxemia	Anoxia Vagal stimulation Anesthetic poisoning of respiratory enzymes Decrease cardiac output Potassium inhibition Adrenal cortical insufficiency, etc	Terminal asystole	Inflate lungs Increase oxygen in anesthetic mixture Intravenous oxygen Transfusion of oxygenated blood Cytochrome Cardiac massage ? Cardiac stimulants, etc
Sinus or nodal tachycardia Auricular or nodal premature systoles Minor aberration of QRS		Arrhythmias of no clinical significance	

two extremes lies the real importance of the electrocardiogram during anesthesia and operation: the early detection of abnormalities of the cardiac mechanism which indicate serious myocardial damage and which might progress to a terminal arrhythmia. It is hoped that

such knowledge of the heart's action might lead to intelligent and effective management with the resultant saving of lives which might be lost otherwise

The first problem encountered in a study of this sort is the determination of just what constitutes a significant abnormality of the cardiac mechanism. These may be classified as follows

Electrocardiographic

- I Major disturbances of heart rate
 - A Ectopic tachycardia
 - B Sinus or AV nodal bradycardia of extreme degree, especially with failure to revert to and remain normal after atropine
- II Evidence of anoxemia
 - A ST segment and T wave abnormalities
 - B Major disturbance of intraventricular conduction (bundle branch block)
 - C Complete AV dissociation (exclusive of interference dissociation)
 - D Sinus arrest
- III Ectopic arrhythmias with potential danger of ventricular fibrillation
 - A Ventricular premature systoles
 - B Ventricular tachycardia

Direct observation

- I Dilatation and mechanical asystole
- II Increasing cyanosis
- III Signs of cerebral damage (in absence of occlusion of innominate or carotid artery)
- IV Fall in blood pressure without hemorrhage or significant change in heart rate

There appears to be a very wide range of heart rate within which normal cardiac efficiency and normal circulatory dynamics may be maintained. Even the extremes of sinus bradycardia and tachycardia seldom exceed this wide margin of safety. It seems probable that under ordinary circumstances there is little danger of congestive heart failure or peripheral vascular collapse with subsequent vascular thrombosis (cerebral or at the anastomotic site) until the heart rate

exceeds 180-200, and then only when the tachycardia is maintained over a fairly long period of time (probably several hours or more). This circumstance apparently only occurs with an ectopic tachycardia such as paroxysmal auricular or nodal tachycardia (Fig. 3), auricular flutter, or auricular fibrillation, and constitutes an indication for prompt and adequate treatment. Effective treatment consists primarily of vagus nerve stimulation, either reflexly by carotid sinus or ocular pressure, or directly by specific medication such as the administration of mechoyl. Since mechoyl and related drugs have several unpleasant side-effects and at best may not be given without significant danger, and because carotid sinus pressure alone is frequently ineffective, the intravenous administration of a purified glycoside of digitalis appears to be the treatment of choice. This may be given as lanatoside C or digitoxin in a dosage of approximately 30 micrograms per kilogram of body weight. Atropine, by preventing the effects of vagal stimulation, may tend to perpetuate this type of arrhythmia at least until the effects of its previous administration have subsided. This constitutes one possible objection to the routine preoperative use of atropine especially in individuals who give a history of paroxysmal tachycardia or who may be predisposed in any way to the development of such an arrhythmia. Bradycardia, on the contrary, usually responds to atropine by a prompt reversion to normal sinus rhythm or sinus tachycardia. Consequently, when the heart slows or when nodal rhythm with or without interference dissociation occurs as a result of the anesthetic or of vagal stimulation, the administration of atropine is indicated. When the ventricular rate is less than 50 per minute and when the bradycardia fails to revert to normal or tends to reoccur after the administration of atropine, the danger of a terminal cardiac arrhythmia may be considered to be imminent and the institution of well-considered therapeutic procedures urgent.

Until the exact pathological physiology of these preterminal arrhythmias is known, treatment must remain upon a more or less empirical basis. From the observations of the present study, however, a number of suggestions for specific therapy and for further investigation may be made. Thus, there is evidence that severe anoxemia may be a factor of considerable importance in the development of a serious degree of myocardial depression (13). Further detailed information on this

problem should be derived from a correlation of electrocardiographic data with direct oximeter determinations of arterial or capillary oxygen saturation during anesthesia and operation. Until such a study is made it would seem reasonable to assume that pulmonary collapse and the unavoidable interruption of collateral circulation incident to thoracotomy and mediastinal dissection must almost certainly reduce an already decreased arterial oxygen saturation to dangerously low and perhaps lethal levels despite the original slight increase which may occur during induction (12). If such is the case, a number of therapeutic procedures may be considered. The first and simplest would be to increase the availability of oxygen. However, under the circumstances described, breathing even 100% oxygen under positive pressure will probably help little if at all because of the marked reduction of pulmonary circulation which is present until after the newly created systemic-pulmonary anastomosis has been opened and the collapsed lung re-expanded. It follows that even before completion of the anastomosis periodic inflation of the collapsed lung might be of benefit by making the oxygen in the anesthetic mixture more available to the pulmonary circulation, particularly in individuals considered to be especially vulnerable from the standpoint of anoxia. (The frequency of re-expansion of the lung could be determined perhaps by oximeter readings.) An alternative method of making oxygen available, but one which has not proved to very practicable as yet, is to administer gaseous oxygen intravenously (34) or to transfuse the patient with oxygenated blood (14). An entirely different solution to the problem is offered in the use of respiratory enzymes and related materials such as cytochrome C (23), the B vitamins (24), and perhaps others, for increasing tissue utilization of oxygen and possibly helping the patient thereby to survive a relatively brief period which might otherwise terminate fatally. It must also be remembered that tissue anoxia may be stagnant or anemic rather than, or in addition to, anoxic. The factor of anemia is not uncommon, particularly in infants, and should be corrected preoperatively by proper methods such as the administration of iron and small repeated blood transfusions. During anesthesia and operation stagnant anoxia, which may result from a failing circulation and which may in turn further depress the heart and circulation, should be benefited by the action of cardiac stimulants such as epi-

nephrene and digitalis Adequate attention must be given, in addition, to other factors known to be important in thoracic surgery, both cardiac and non-cardiac, including the maintenance of an adequate airway, control of the movement of the lung and mediastinum, the prevention of too great a degree of dependency of the good lung during open pneumothorax, the prevention of vascular obstruction by improper retraction, and others (2)

Should the heart dilate and stop, two questions must be answered (1) What methods must be used for immediate resuscitation? and (2) If the heart can be revived, what should be done about continuing or not with operation? The subject of resuscitation of the failing heart is still a controversial one, although certain procedures are generally accepted and certain others warrant further consideration (1, 5, 6) There appears to be a sound basis for the immediate institution of cardiac massage—not just plain “squeezing” of the heart—but the rhythmical application of external force to the ventricles in a direction from apex to base in order to simulate as closely as possible the normal action of the heart and thereby to maintain at least temporarily an adequate circulation (5) It must be remembered also that the maintenance of artificial respiration with a high concentration of oxygen is also necessary throughout this entire period Manual massage probably accomplishes little more than the artificial maintenance of the circulation until such time as the heart can resume its own rhythmical action either spontaneously or more often with the help of other supportive and stimulative measures The intracardiac injection of epinephrine has been one of the most widely recommended procedures (5, 7, 27), but there remains a great deal of controversy about the advisability of its use Its effectiveness has been proclaimed by some and disclaimed by others The danger of precipitating fatal ventricular fibrillation especially during cyclopropane anesthesia has been emphasized many times (19, 20, 22) and cases cited in which this was the “probable” cause of death It is to be noted, however, that electrocardiographic evidence of this cardiac mechanism as a cause of death has been lacking in most human cases and, further, that in the present study ventricular fibrillation never occurred even after the administration of large amounts of epinephrine This should not be considered final proof against the theory of cyclopropane sensitization of the heart to epi-

nephrine and the resulting danger of ventricular ectopic arrhythmias (19, 22) It may indicate simply that such sensitization does not occur in this particular type of case, in which severe anoxia may act as a general cardiac depressant instead of inducing localized regions of increased irritability, as in diseases of the coronary arteries The observations cited in this study, as well as those of other recent publications (13, 21, 29) tend to support such a concept Although it still remains a matter of some difference of opinion, it seems reasonable to state, for the present at least, that the use of epinephrine and other cardiac stimulants is of questionable and poorly defined value It is likely that, although they may increase the force of contraction when the heart is still beating, however feebly, they will not often initiate contractions after they have already ceased Greater value might be expected from the administration of a longer acting specific cardiac stimulant, such as digitalis, or by supplying the heart with essential materials for energy production, such as available oxygen, respiratory enzymes, electrolytes, etc, at the same time that the circulation is maintained by manual massage or the rhythmical stimulation of an artificial pacemaker

Should normal rhythmicity of the heart be re-established following the occurrence of cardiac asystole or other serious preterminal arrhythmia, the difficult question of continuing operation must be decided It is not possible, nor is it intended, to attempt making any final answer at the present time, for the decision must be made on the merits of each individual case However, several observations are worthy of mention It should be noted, for example, that few children ultimately survive the occurrence of pre-terminal arrhythmias as observed in this study Only three children of the entire group survived operation, and of these two died within 24 hours of cerebral thrombosis which probably occurred during cardiac asystole Thus, only one child ultimately survived, and in this instance no actual surgery was performed since the arrhythmia occurred during anesthesia and operation was indefinitely postponed It seems apparent, therefore, that regardless of proceeding with operation or not, ultimate survival after the occurrence of a preterminal arrhythmia is not likely even after the restoration of apparently normal heart action It is possible, however, that newer methods of treatment may favorably affect this

practically hopeless outlook. Considering the problem from another point of view, to subject a child with congenital heart disease and cyanosis to exploratory thoracotomy with the attendant disruption of a variable but significant number of collateral vessels, without completing the anastomosis for which the operation was intended, does a great deal of harm and frequently results in death. In fact, the mortality rate in such cases is as high or higher than in the group of children in whom the entire operation has been completed. Therefore, unless operation is discontinued or postponed for a specific reason, or unless it can be attempted again in the near future with at least equal or perhaps greater safety, considerable thought must be given to the advisability of discontinuing or of proceeding with operation after the occurrence of the type of arrhythmia under discussion. Frequently the attitude of "nothing to lose and everything to gain" seems justified. Also, it may be that with the development of newer methods of treatment, based upon such principles as those suggested, a more satisfactory answer may be found for this entire problem.

Another type of arrhythmia which requires consideration is the occurrence of frequent premature systoles particularly of ventricular origin. These appear to be due in the great majority of instances to the direct action of cyclopropane on the heart (Fig 9), and have been considered, correctly or not, to constitute a prefibrillary arrhythmia (19, 20, 22). Despite the fact that ventricular fibrillation was never observed in this series, and despite the lack of electrocardiographic evidence to confirm ventricular fibrillation as a major cause of immediate anesthetic death in humans, it appears to be a simple and reasonable measure of security to stop cyclopropane as an anesthetic agent and continue with ether and oxygen in the presence of multiple ventricular premature systoles or ventricular tachycardia. Such a procedure has resulted in the disappearance of the former in every instance, the latter only having occurred in the terminal group. A number of other means have been suggested for preventing ventricular fibrillation. These include the use of barbiturates and other drugs (3) and the local application or parenteral administration of local anesthetics such as procaine (9, 18). There would appear to be little use for this type of therapy except perhaps in ventricular tachycardia, though even in this arrhythmia the danger of ventricular fibrillation

is not great in patients with anoxemia and evidence of cardiac depression. Methods have been tried and recommended also for the management of actual as well as imminent ventricular fibrillation (6).

The other disturbances in the cardiac mechanism observed during this study are largely of academic interest and do not seem to have any immediate clinical significance. This applies particularly to the various forms of nodal rhythm. Of particular interest are the changes which occur in the configuration of QRS without evidence of bundle branch block, primarily those involving gross changes in the position of the mean electrical axis of QRS. This problem is the subject of further investigation, the results of which will be published separately (35). However, certain comments may be made here on the basis of present information. The position of the mean electrical axis in the extremity lead electrocardiogram until recently has been considered evidence of hypertrophy of one or the other ventricle. Even now it is not generally appreciated that other factors, such as the position of the heart in the thorax, may influence even more strongly the configuration of the ventricular deflections particularly in the extremity leads. In the cases under consideration, the position of the heart being relatively fixed and changes in ventricular preponderance obviously not being a factor, a third influence must be considered, namely changes in intraventricular conduction exclusive of those producing gross bundle branch block. The clinical significance of these changes is not at all certain, although they have been described previously in infectious diseases (28) presumably as the result of toxic myocarditis, and in these surgical cases may represent additional evidence of myocardial damage due to anoxemia. It is of interest and probably of some significance that the three children in whom the most marked changes in QRS occurred all had more complicated malformations than a simple tetralogy of Fallot, that all three had severe congestive heart failure following the systemic-pulmonary anastomosis, and that two died in less than one year after operation, the third surviving perhaps only because of ligation of her artificial ductus.¹ Whatever the pathogenesis, the observation that gross alterations in the form and direction of QRS may occur without changes in the position of the heart or changes

¹ These three cases will be reported in further detail elsewhere, the first two by Ziegler (35), the third by Dr William Adams.

in the relative mass of either ventricle and without gross abnormalities of intraventricular conduction, such as bundle branch block, is of considerable importance among the fundamental principles upon which the interpretation of the electrocardiogram is based

CONCLUSIONS

Electrocardiograms were recorded practically continuously during anesthesia and operation in 175 children with congenital heart disease and cyanosis. From this study the following observations concerning the occurrence, significance, and management of abnormalities of the cardiac mechanism were made

- 1 Arrhythmias of some sort occurred in approximately 80% of the entire group of patients. These varied from simple variations of rate to complex terminal mechanisms and included many forms of active and passive ectopic arrhythmias which occurred throughout all phases of anesthesia and operation.

- 2 The most common abnormality was the group of arrhythmias resulting from depression of the normal pacemaker with supercedence of some center of lower rhythmicity, usually the atrio-ventricular node. This abnormality of the cardiac mechanism was usually benign and in most instances reverted to normal, either spontaneously or following the intravenous administration of atropine.

- 3 Arrhythmias resulting from increased cardiac irritability, including primarily ventricular premature systoles and paroxysmal auricular or AV nodal tachycardia, occurred in slightly more than 10% of the patients, and, although these may be considered generally to be relatively benign disorders of the cardiac mechanism, under the circumstances of cyclopropane anesthesia and intrathoracic operation in a child with an abnormal heart and anoxemia they were treated with certain potential dangers in mind. Ventricular premature systoles were believed to constitute a possible precursor to a much more serious ventricular tachycardia or fatal ventricular fibrillation and responded satisfactorily in all instances to stopping cyclopropane and continuing with ether and oxygen or oxygen alone. Paroxysmal tachycardia, especially with a long continued rapid ventricular rate, was considered potentially hazardous because of the possibilities of congestive heart failure or peripheral vascular collapse, with resulting venous or arterial

thrombosis, and was treated immediately by vagus stimulation, usually in the form of the intravenous administration of digitalis in full dosage

4 Spontaneous changes in the form of the ventricular deflections, detectable only in the electrocardiogram, appeared to have no immediate significance from the surgical viewpoint, but seemed to occur more frequently in children with more complicated cardiovascular malformations than the simple tetralogy of Fallot and whose post-operative course was commonly complicated by the occurrence of congestive heart failure

5 The terminal cardiac mechanism was recorded in seven children who died during operation, and in each instance the final event was sinus arrest with complete cardiac asystole. On the basis of a careful analysis of the entire electrocardiographic record it was believed that in practically every instance terminal asystole was preceded by one or more specific preterminal phenomena including the following: sinus or AV nodal bradycardia with a rate of less than 55 per minute and either failing to revert to normal or recurring after the administration of adequate amounts of atropine, complete AV dissociation with idioventricular rhythm, ventricular tachycardia, complete bundle branch block, and other electrocardiographic changes interpreted as evidence of myocardial anoxia. Other evidences of hypodynamic heart action with impending terminal asystole included falling blood pressure, not the result of hemorrhage or significant change in heart rate, progressive decrease in arterial oxygen saturation, and mechanical dilatation of the heart with asystole (occasionally with a normal electrocardiogram)

6 Evidence was given for believing that the terminal cardiac mechanisms are manifestations of myocardial depression as a result of anoxia. On the basis of this assumption treatment should be directed logically toward the correction of anoxia in all of its characteristic manifestations, including primarily the provision of available oxygen to the tissues and support for the failing heart and circulation. The value and the limitations of specific procedures were discussed. Although it is not possible at present to determine at what point the preterminal cardiac mechanism becomes irreversible, it seems certain that the earlier specific treatment is instituted the more likely it will be to result successfully, and, as in many other circumstances, prevention is more important than any treatment.

7 It is of significance that ventricular fibrillation never occurred as a cause of death even following the injection of epinephrine during cyclopropane anesthesia, thus providing further indirect evidence for the belief that anoxemia is a myocardial depressant. It seems apparent that epinephrine may be given with relative safety in the conditions of this study. It is probably true, however, that the administration of this drug is of little if any value in initiating cardiac contractions after they have already ceased.

8 Some indication has been given that digitalis may be of great value in maintaining normal cardiac function during anesthesia and operation, especially in selected patients. Additional investigation will be necessary to support this as well as certain other concepts encountered in this study.

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ADDENDUM

Since the completion of this electrocardiographic study, a continuous, systemic, arterial oxyhemoglobin concentration record was made by the method of Hartman, et al,¹ during operation on a twenty-four year old patient with tetralogy of Fallot and functional complete pulmonary atresia. This case will be reported in further detail elsewhere but certain observations should be mentioned in connection with this study. These include the following:

1 The spontaneous occurrence of "hypodynamic heart action" with fall in blood pressure, imperceptible pulse and progressive decrease in oxygen saturation immediately following induction of anesthesia.

2 Beneficial but transient effect of epinephrine as evidenced by a return of oxygen saturation to the pre-anesthetic level.

3 Immediate and prolonged beneficial effect of a sub-digitalizing amount (two-thirds of the calculated dose) of intravenous digitals as evidenced by systemic arterial oxygen saturation, blood pressure and heart action.

4 Maintenance of constant oxygen saturation in the face of open pneumothorax with its attendant complete atelectasis and with clamping of the pulmonary artery.

5 Immediate post-operative rise of oxygen saturation to normal after releasing the clamp at the anastomotic site.

It will be seen that these observations support, in part, the principles

¹Hartman, F W, Behrmann, V G, and Chapman, F W Photo-electric Oxyhemograph, a continuous method for measuring the oxygen saturation of the blood *Am J Clin Path*, 18 1-13, Jan 1948

previously mentioned regarding the relationship between cardio-circulatory failure and anoxemia. It is evident also on the other hand that oxygen saturation in the systemic blood need not necessarily decrease as the result of collapse of a lung and the disruption of collateral circulation. The results of this single study confirm the need for further observation of this sort which should contribute significantly to the solution of many of the problems encountered.

OBSERVATIONS ON THE EFFECTS OF FOLIC ACID ANTAGONISTS, FOLIC ACID, LIVER EXTRACT AND VITAMIN B₁₂ ON EMBRYONATED EGGS¹

A PRELIMINARY REPORT

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This is a preliminary report of observations made on embryonated eggs following injections of three folic acid antagonists (1, 2), folic acid, purified liver extract and vitamin B₁₂

Six to 8-day old chick embryos were inoculated via the yolk sac with three folic acid antagonists (4-amino-pteroylglutamic acid, N¹⁰-methyl-pterioic acid and methyl-4-amino-pteroylglutamic acid)⁴ alone or with folic acid (synthetic pteroylglutamic acid),⁴ purified anti-pernicious anemia liver extract, or vitamin B₁₂⁵ in the amounts indicated in the table All substances except the liver extract were dissolved in distilled water The embryos were incubated at 35°C All deaths occurring in the first 48 hours were considered post-traumatic and discarded from the study Yolk sacs were fixed in 10 per cent acetic acid Zenkers solution and microscopic sections were stained with eosin-methylene blue⁶

The findings are recorded in Table I Embryonated eggs injected with 4-amino-pteroylglutamic acid in amounts of 0.005 mg showed a

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² Research Fellow of the American College of Physicians

³ Senior Fellow in Medical Sciences, National Research Council

⁴ Through the courtesy of the Lederle Laboratories, Pearl River, N Y the following materials were provided folic acid as synthetic pteroylglutamic acid, 4-amino-pteroylglutamic acid (Lot #7-7843C), N¹⁰-methyl-pterioic acid (Lot #7-7623A), and methyl-4-amino-pteroylglutamic acid (Lot #7-8185)

⁵ Vitamin B₁₂ was provided through the courtesy of Dr A Gibson of Merck & Co, Inc, Rahway, New Jersey

⁶ The authors are indebted to Miss Lillian M Leavitt of the Mallory Institute of Pathology for preparing the microscopic sections

TABLE I
Effect of Folic Acid Inhibitors on Chick Embryos

SERIES	DRUG	DOSE	AGE AT INOCULATION	SURVIVAL TIME IN TERMS OF DAYS OF AGE										APPEARANCE OF YOLK SAC BLOOD ISLETS
				6	7	8	9	10	11	12	13	14		
1	4-amino-pteroyl-glutamic acid	0.005 mg	8 days			5/5*	5/5	5/5	5/5	3/5	1/5	0/5	Diminution in size and number of blood islets Pyknosis, karyolysis karyorrhexis of nuclei Same as Series 1	
2	4-amino-pteroyl-glutamic acid	0.005 mg	8			3/3	3/3	3/3	3/3	2/3	2/3	0/3	Same as Series 1	
3	Vitamin B ₁₂ † 4-amino-pteroyl-glutamic acid	0.005 mg 0.005 mg	7		13/13	13/13	13/13	11/13	6/13	0/13			Same as Series 1	
4	Liver extract† 4-amino-pteroyl-glutamic acid	3.75 units 0.005 mg	7		16/16	16/16	16/16	16/16	15/16	12 3/16	11/16	10/16	Slight diminution in cellularity of blood islets	
5	Folic acid† N ¹⁰ -methyl-pteronic acid	12.5 mg 20 mg	6	8/8	8/8	8/8	7/8	6/8	4/8¶	4/8			Normal	
6	Methyl-4-amino-pteroylglutamic acid	0.01 mg	8			11/11	11/11	11/11	11/11	11/11	7/11	6/11	Diminution in size and number of blood islets Fragmentation of nuclei	
7	Methyl-4-amino-pteroylglutamic acid Folic acid†	0.01 mg 0.1 mg	8			12/12	12/12	12/12	12/12	11/12	10/12	10/12	Slight diminution in cellularity of blood islets Occasional fragmentation of nuclei	

* No survived/No injected † Injected at same time ‡ Injected 24 hours before antagonist § 3 eggs sacrificed for study

¶ 2 eggs sacrificed for study || Experiment terminated

decreased survival time (Series 1) The blood islets from the yolk sacs of such embryos were diminished in both number and size Frequently the nuclei of the hemopoietic cells in such areas were pyknotic or showed karyolysis and karyorrhexis

Injection of 0.005 mg of vitamin B₁₂ simultaneously with the inhibitor (Series 2) did not prolong the survival time or modify the histological appearance of the blood islets As much as 3.75 USP units (injectable) of purified liver extract (containing the equivalent activity of 2.7 micrograms of vitamin B₁₂) injected 24 hours prior to the administration of the 4-amino-pteroylglutamic acid (Series 3) did not prevent a shortened life span or alteration in the appearance of the yolk sac blood islets However, when folic acid was given in 12.5 mg amounts 24 hours prior to the antagonist (Series 4) the blood islets were not decreased in number though their cellularity was somewhat diminished Pyknosis, karyolysis and karyorrhexis of the nuclei were not present There was a suggestive increase in survival time

N¹⁰-methyl-pterotic acid in 20 mg amounts under the conditions of the experiment produced no detectable alteration of the blood islets (Series 5)

Embryonated eggs injected with methyl-4-amino-pteroylglutamic acid in amounts of 0.01 mg showed a decrease in the number and size of yolk sac blood islets with some fragmentation of the nuclei of the hemopoietic cells (Series 6) These changes were not as marked as those occurring after the use of 4-amino-pteroylglutamic acid The simultaneous injection of 0.1 mg of folic acid (Series 7) resulted in less marked cytological changes in the blood islets There was a slight decrease in the cellularity of the islets and occasional fragmentation of nuclei

SUMMARY

A new method of study of the effects of folic acid inhibitors is described Of the 3 antagonists studied, 4-amino-pteroylglutamic acid was the most effective in small doses There were marked cytological changes in the yolk sac blood islets, characterized by diminution in their size and number with pyknosis, karyolysis and karyorrhexis of many of the remaining nuclei There was a suggestive shortening of the survival time of the embryos This effect, as well as that of the

less active methyl-4-amino-pteroylglutamic acid, appeared altered by injections of folic acid but not by liver extract in the doses employed. Vitamin B₁₂, in the amount used, did not alter the apparent effect of 4-amino-pteroylglutamic acid. N¹⁰-methyl-pteronic acid in relatively larger doses than the other antagonists caused no detectable changes in the yolk sac blood islets.

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THE EFFECT OF OVARECTOMY AND PHYSIOLOGIC DOSES OF ESTRADIOL UPON BODY WEIGHT, LINEAR GROWTH AND FAT CONTENT OF THE FEMALE ALBINO RAT

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INTRODUCTION

It is well known that large doses of estrogen will retard normal weight gain and body growth of experimental animals. The purpose of this experiment is to see whether small doses of estrogen comparable to the natural supply will also cause delay in growth and weight gain of rats, and to determine whether this effect is in part due to alteration in the amount of body fat. The effect of these small doses of estrogen upon the reproductive organs and upon the blood level of calcium, phosphorus and phosphatase was also determined.

METHODS

Preliminary experiments showed that estradiol benzoate in a dosage of 30 micrograms weekly markedly retarded the weight gain of young female albino rats. Half of this dose was used in this experiment. Five micrograms of estradiol benzoate dissolved in 0.1 ml of peanut oil were injected three times a week. The total amount of estradiol administered over the 40 day period was 85 micrograms or about 2.1 micrograms daily.

Four groups of ten female albino rats of approximately the same age and weight were observed: a control group, an ovariectomized group, and two similar groups treated with estradiol. Treatment was commenced after vaginal opening when the rats were 42-44 days old and weighed approximately 85 grams. The ovariectomized animals were allowed four days to recuperate from the operation and ether anesthesia before the treatment period was instituted.

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Food and water were available at all times. The diet consisted of fox chow pellets supplemented with lettuce daily and carrots once a week. The animals were weighed at five day intervals and measured before and after the treatment period. The carcasses were x-rayed at the conclusion of the experiment.

At the start of treatment vaginal smears were taken daily, later at frequent intervals. At the conclusion of the treatment period the animal was weighed again and measured and then anesthetized with nembutal. Blood was taken from the heart and then the animal was killed and skinned. The adrenals, ovaries and uterus were

TABLE I

GROUP	WEIGHT GAIN GRAMS			LINEAR GROWTH CMS			UTERUS WEIGHT MGS /100 GMS			OVARY WEIGHT MGS /100 GMS			TOTAL FAT GMS /100 GMS		
	Mean	Difference	S.E.*	Mean	Difference	S.E.	Mean	Difference	S.E.	Mean	Difference	S.E.	Mean	Difference	S.E.
Control	73.3			14.4			198			59			5.2		
Castrate	119.0	45.7	7.0	16.8	2.4	0.6	31	-167	28				4.9	-0.3†	0.6
Control	73.3			14.4			198			59			5.2		
Estradiol	53.6	-19.7	4.8	12.0	-2.4	0.7	293	95	40	38	-21	5	6.4	+1.2	0.4
Control	73.3			14.4			198			59			5.2		
Castrate Estradiol	69.4	-3.9†	5.2	12.3	-2.1	0.6	202	4†	28				7.3	+2.1	0.5

* S.E. or standard error of the difference of the means = $\sqrt{SE_m^2 + SE_m^2}$ where $SE_m = \sqrt{\frac{\epsilon \Delta^2}{n(n-1)}}$

† Not a statistically significant difference

weighed and fixed. The hypophysis was also removed for histological study.

The skin and carcass were dissolved separately in four times their weight of 50 percent potassium hydroxide in 50 percent ethyl alcohol. After four hours of heating, the mixture was acidified with 50 percent sulfuric acid and extracted with petroleum ether. The extract was dried with sodium sulfate and filtered. The filtrate was evaporated to dryness and weighed.

RESULTS

The weight gain of the four groups of animals is shown in Table I. The average gain of the untreated animals was 73 grams in forty days.

while the ovariectomized rats gained 119 grams, which was 46 grams more than the controls. The average gain of the estradiol treated animals was 54 grams, or 20 grams less than the controls. The ovariectomized rats which were treated with estradiol gained slightly less than the controls, but the difference was not statistically significant (Chart I).

The growth of the tail exceeded the growth of the body in all groups, but the difference between body growth and tail growth was small and in proportion to the total growth. The normal rats grew 14.4 cms in length during the forty days. The ovariectomized rats grew 16.8 cms, an increase over normal of 2.4 cms. The estradiol treated rats grew 12.0 cms, or 2.4 cms less than the controls. The ovariectomized and estradiol treated animals grew 12.3 cms, which is not significantly different from the other estradiol treated group but which is significantly less than the normal controls (see Table I). The differences in skeletal growth are illustrated by x-rays, Figure 1.

The uterine weight of the normal rats averaged 198 mgs per 100 gms body weight. Ovariectomy reduced this figure to 31 mgs, while treatment with estradiol raised it to 293 mgs. The group that was castrated and treated with estradiol had an average uterine weight of 202 mgs per 100 gms body weight. The ovaries in the estradiol treated rats weighed only 38 mgs per 100 gms body weight compared with 59 mgs in the controls.

The total amount of fat in the skin and carcass was greater in the ovariectomized rats than in the controls, but the percent of fat in relation to the weight of the skin and carcass was lower than that of the controls. The fat in the body of the estradiol treated rats was greater in amount and percent than that of the controls although the actual difference was small, Table II. The rats that were ovariectomized and treated with estradiol showed the greatest amount and the greatest percent of fat of all groups.

The serum calcium of the pooled sera of the normal rats averaged 9.9 mgs percent compared with 10.5 and 10.8 in the two groups treated with estradiol and 9.8 in the ovariectomized animals, see Table III. The average serum phosphorus level of the normal rats was 10.1 mgs percent compared with 12.6 in the ovariectomized group and 11.9 and 10.2 in the estradiol treated animals. The serum alkaline phosphatase

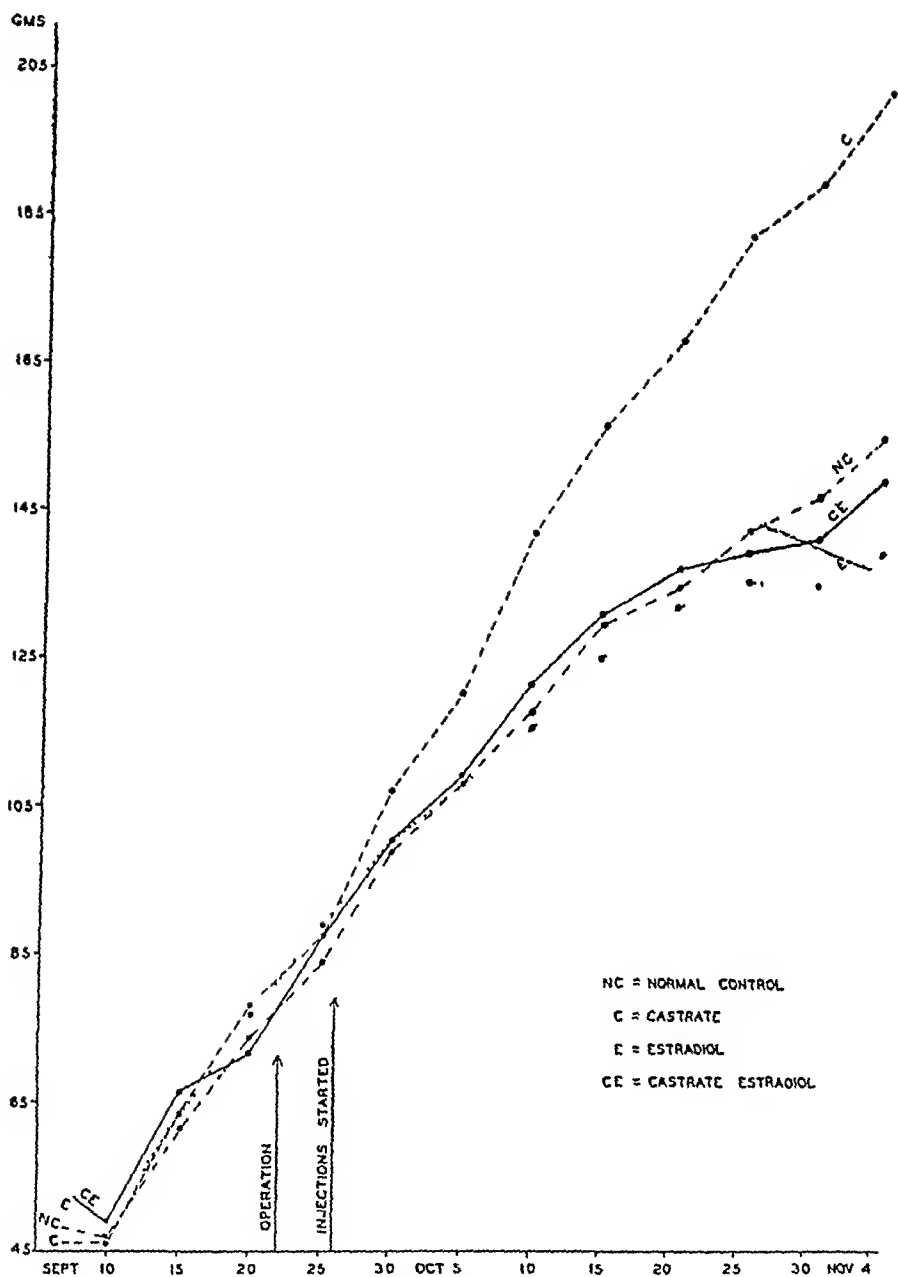


CHART I

expressed in Bodansky units per 100 cc was 88 in the normal rats. A considerably higher value, 105, was found in the pooled blood serum of the ovariectomized rats, and a somewhat lower value was found in the two groups treated with estradiol, 69 and 71.

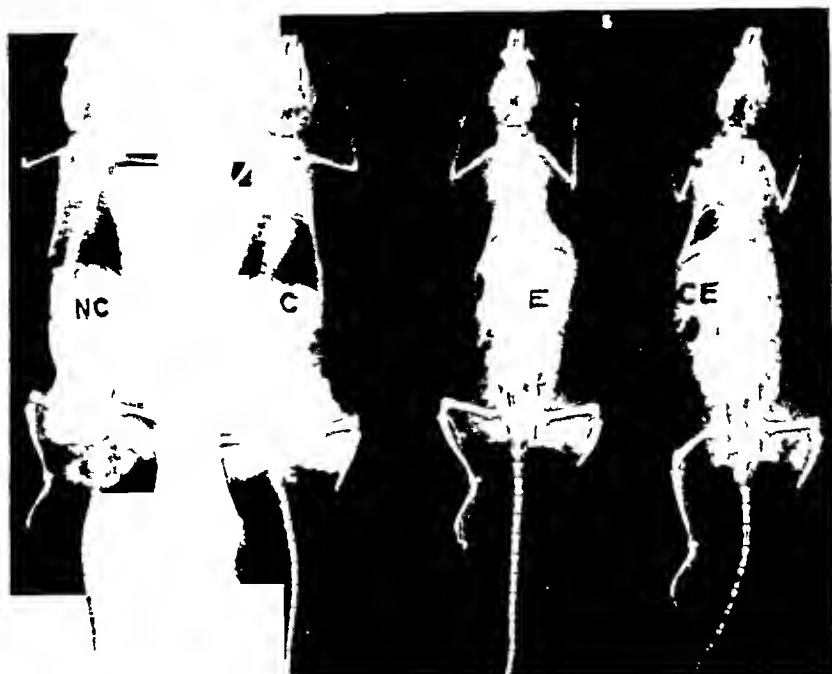


FIG 1

NC = Normal Control

C = Castrate

E = Estradiol

CE = Castrate Estradiol

TABLE II

GROUP	SKIN			CARCASS			TOTAL ANIMAL		
	Weight	% Fat	Total Fat	Weight	% Fat	Total Fat	Weight	% Fat	Total Fat
Normal	21.2	11.7	2.48	138	4.2	5.77	158	5.2	8.25
Castrate	29.3	10.8	3.20	178	3.9	6.96	208	4.9	10.16
Estradiol	18.3	13.6	2.48	128	5.2	6.70	143	6.4	9.18
Castrate Estradiol	18.9	14.8	2.79	137	6.4	8.82	158	7.3	11.61

TABLE III

GROUP	CALCIUM mg%	PHOSPHORUS mg%	PHOSPHATASE* B U /100cc
Normal	9.9	10.1	88
Castrate	9.8	12.6	105
Estradiol	10.5	11.9	69
Castrate Estradiol	10.8	10.2	71

* Alkaline phosphatase expressed in Bodansky units

Vaginal smears taken on the control group showed a normal estrus cycle of approximately four days. The ovariectomized animals were in constant diestrus. The rats receiving estradiol were in constant estrus, although occasionally the ovariectomized rats receiving estradiol showed smears suggesting proestrus.

Histological study of the organs showed that, although there were variations in size, the structure of the adrenals was the same in all groups. The liver appeared normal in all the animals. The ovaries in the rats treated with estradiol showed the presence of many small and large cysts despite the decrease in size. The uterus was almost completely atrophied in the ovariectomized animals. In the rats treated with estradiol, both the normal and the ovariectomized groups, the uterus was normal or enlarged with prominent glands showing an increased number of vacuoles.

DISCUSSION

The fact that the ovariectomized rats gained weight more rapidly than the normal animals is in agreement with the results of other observers. Holt, Keeton and Vennesland studied ovariectomized rats and found that they had a larger food intake than normal rats of the same age (1). This might account in part for accelerated gain in weight.

Levie has shown that the administration of estrone in doses of 250-500 micrograms daily markedly suppresses gain in body weight, although in doses of 10-20 micrograms the reduction in weight gain was slight and inconstant (2). However, estrone is known to be biologically less active than estradiol. Hooker and Pfeiffer, using estradiol benzoate, found that 83 micrograms administered twice weekly, which averages 24 micrograms daily, interfered with normal weight gain (3). It is remarkable that the dose of estradiol benzoate used in this experiment, 5 micrograms three times a week, which averages 2 micrograms daily, was sufficient to retard the weight gain of normal rats and to completely counteract the increased weight gain usually shown by ovariectomized rats.

It was pointed out many years ago by Stotsenburg that in mammals the male is usually larger than the female (4) and Holt was able to produce females of larger than normal size by ovariectomy (1). The

ovariectomized rats in our experiment showed a marked increase in linear growth over that of the normals. X-ray studies showed that there was a general increase in the size of the bones affecting the width of the skull and pelvis, as well as the length of these and the long bones.

Levie found no alteration in bone growth when doses of 5-20 micrograms of estrone were given although a definite effect was obtained with 250-500 micrograms daily (2). In the studies reported here, a dosage of 5 micrograms of estradiol benzoate three times a week, which averages 2 micrograms daily, caused a stunting of growth in normal rats of the same degree as the increase in growth shown by the ovariectomized animals. The rats that were ovariectomized and given estradiol showed the same skeletal growth as the normal animals that were treated with estradiol, showing that this dose was more than enough to counteract the effect of ovariectomy upon bone growth.

It is interesting to note that the uterus in the ovariectomized rats which were given 2 micrograms of estradiol daily was almost exactly the same size as the uterus in the control animals. The uterus in the normal rats that were treated with estradiol showed considerable enlargement beyond that of the normal. The fact that the 2 micrograms daily of estradiol approximately duplicated in ovariectomized animals the degree of uterine development which occurred in the controls does not prove that this dosage was equivalent to the normal physiological output of estrogen, because the action of estrogens may be modified by other steroids, such as progesterone.

It is frequently stated that ovariectomy causes an increase in obesity similar to that which follows castration of the male (4). However, quantitative studies showed that this increase in fat was proportional to the increase in body weight and that the percentage weight of fat was not higher in the ovariectomized animals. Holt (1) analyzed the fat content of rats twelve months after ovariectomy and found no increase compared to normal controls of the same age.

On the other hand it has been surmised that the diminished weight gain caused by the administration of estrogen might be due to a reduction in the fat deposits. However, in these experiments there was found to be a slight increase in the fat content of the estradiol treated animals, which was more striking when compared with the body weight

since these animals were the lightest group. The rats which were ovariectomized and given estradiol had the greatest amount of fat and the greatest percentage of fat. These observations are in harmony with the general observation that the female of the species has a higher percent of body fat than the male (5).

The rise in serum calcium observed in the estradiol treated rats is quite small compared with the rise observed by Pfeiffer and Gardner in birds receiving large doses of estradiol benzoate (7). The changes in serum phosphate were not as striking as the changes in alkaline phosphatase. The fact that the highest value of phosphate and phosphatase occurred in the ovariectomized group is compatible with the observation that phosphate and phosphatase are increased during the period of growth. It has been suggested that the level of serum phosphate is an indication of the production of growth hormone, and it has been shown by Albright and co-workers that estrogen diminishes the level of serum phosphate and bone growth in acromegalics (8). This is not surprising since in 1936 Zondek showed that in rats the administration of estrogen arrested growth through its effect upon the hypophysis (9). It is notable that while the phosphate level was higher in the ovariectomized rats it was not lowered by the administration of estradiol although the serum alkaline phosphatase was depressed. A depression in the serum alkaline phosphatase with estrogen treatment was noted by Day and Follis who studied the composition of rat bones after prolonged treatment with estradiol (10).

It may be concluded that, when given in physiological doses, the effect of estrogen upon weight gain is manifested chiefly through its inhibition of growth. With the decrease in growth there is a moderate increase in the proportion of body fat. This is opposite to the effect of androgen which causes an increase in growth and a decrease in the relative amount of fat (5). Furthermore, although a dose of 5 micrograms of estradiol benzoate given three times a week is just sufficient to maintain normal uterine weight, it markedly suppresses the normal skeletal growth.

SUMMARY

Female rats ovariectomized at the age of forty days showed a greater increase in both skeletal length and body weight during the

next forty days than did normal female controls of the same age there was a slight decrease in the proportion of body fat

The administration of estradiol benzoate in a dose of 5 micrograms Three times a week to female rats of this age caused a marked retardation of skeletal growth, a diminution in the rate of weight gain, and a considerable increase in the proportion of body fat

Five micrograms of estradiol benzoate administered three times a week to ovariectomized rats was sufficient to maintain the uterus at the same weight as normal controls but inhibited skeletal growth These animals showed a relative and absolute increase in body fat

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CIRCULATING ANTICOAGULANT AS A CAUSE OF HEMORRHAGIC DIATHESIS IN MAN

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Hemorrhagic syndromes associated with defective blood coagulation have almost invariably been attributed to deficiency of one or the other of the plasma components necessary for normal clotting. The spontaneous occurrence of an anticoagulant in the circulating blood has been rarely considered to be a cause of abnormal bleeding in human patients. Quick (11) in his extensive monograph on the hemorrhagic diseases did not mention such a condition. However, several well-studied cases have been reported in which prolongation of the clotting time and bleeding tendency have been associated with the presence of a clotting inhibitor in the circulating blood (2, 3, 4, 6, 8, 12). We have had the opportunity to study three patients in whose blood an anticoagulant has been demonstrated.

ABSTRACTS OF CASES⁵

1 J H, (B C H #114201), a 67 year old senile colored male was admitted to the Baltimore City Hospitals in October, 1947, because of hematuria of several weeks duration. There was no family history of hemorrhagic tendency and no previous history of abnormal bleeding. Genito-urinary study revealed no demonstrable cause of the hematuria. Because the patient had moderate generalized glandular enlargement, a lymph node biopsy was performed. This procedure was followed by profuse bleeding from the wound for several days. Sections of the node showed only hyperplasia. Defective blood coagulation was observed before any transfusions were given. The venous clotting time was 68 minutes. Recalcified plasma clotting time was prolonged to 12 minutes (normal about 2

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⁵ Cases 1 and 2 will be described in detail in subsequent reports

minutes) The prothrombin time was normal Plasma fibrinogen concentration was 0.21 grams per 100 cc Platelet count, bleeding time and capillary fragility were within the limits of normal

2 A V, (J H H #445357), a 39 year old white farmer was admitted to The Johns Hopkins Hospital in December, 1947, because of vague abdominal pain, malaise, and weight loss of three months' duration There was no family history of hemorrhagic diathesis and no past history of abnormal bleeding He had had a herniorrhaphy, appendectomy, and hemorrhoidectomy prior to 1944 without unusual bleeding He had never received a transfusion In 1942 he was found to have a positive serologic test for syphilis for which he was treated In 1944, while in the army, he was hospitalized for trench foot and was reported to have a cardiac murmur and "chronic glomerulo-nephritis" In November 1947 he had two episodes of hemoptysis without other definite pulmonary symptoms Physical examination showed apical systolic and presystolic murmurs and slightly enlarged spleen The urine contained many red cells and variable amounts of albumin A serologic test for syphilis was positive X-ray of the chest showed no abnormality of the heart or lungs While in the hospital he had two more episodes of hemoptysis and occasionally complained of abdominal pain A definite diagnosis was not established Venous clotting time was 60 minutes, recalcified plasma clotting time $7\frac{1}{2}$ minutes, prothrombin time 30 seconds (normal 16 to 20 seconds) The plasma fibrinogen concentration was 0.53 grams per 100 cc Platelet count, bleeding time, and capillary fragility were normal

3 F Y, (J H H #104833), a 38 year old hemophiliac was admitted to The Johns Hopkins Hospital in February 1948 for tooth extraction Several maternal uncles and two brothers had hemophilia The patient had had 10 previous admissions for various hemorrhagic manifestations He had received numerous transfusions, and on his last admission in August, 1947 had been given both blood and anti-hemophilic globulin Venous clotting time was 5 hours, recalcified plasma clotting time 17 minutes, prothrombin time normal Platelet count, bleeding time, and capillary fragility were normal

EXPERIMENTAL STUDIES

Demonstration of the presence of anticoagulant in plasma

Blood was obtained from each of the three patients using silicone-treated needles, syringes and tubes After centrifugation in an angle centrifuge at 5000 to 12,000 RPM for about 20 minutes, the supernatant plasma was removed This plasma was essentially platelet-free and failed to clot even when stored for days in glass tubes In this manner, permanently fluid native plasma was obtained without the necessity of adding anticoagulant to the blood The presence of spon-

taneously occurring anticoagulant was demonstrated by adding small amounts of this plasma to freshly drawn normal human blood and observing the effect on the clotting time. The results of two assays on each of the three patients are shown in Table 1. For comparison there is included in the table an identical study on the plasma of a hemophilic (A B), who had no demonstrable anticoagulant in his blood.

It is apparent that each of the three patients had a potent clotting inhibitor in his plasma. The anticoagulants in the plasmas of J H

TABLE 1

Effect on the clotting time of normal human blood of admixture with plasma from our 3 patients

Patient A B was an ordinary hemophilic without circulating anticoagulant, included for comparison. All clotting times were done by a 3 tube method, the recorded time being that of the third tube. Addition of 0.5 cc. or less of 0.85% sodium chloride solution to 1 cc. normal blood had no effect on the clotting time, therefore, data for the saline controls are not included in the table.

SOURCE OF PLATELET FREE PLASMA	DATE	CLOTTING TIME IN MINUTES OF 1 CC PORTIONS OF NORMAL BLOOD AT 37°C AFTER ADDITION OF PLATELET FREE PLASMA FROM PATIENT VOLUME OF PLASMA ADDED				
		0	0.01 cc	0.1 cc	0.2 cc	0.5 cc
J H	12-10-47	14		39	67	
	3- 1-48	7	32	40	43	
A V	12-29-47	13		39	75	75
	1-27-48	13	20		57	
F Y	2-17-48	14				68
	2-19-48	18		25	31	54
A B	1-13-48	8		9	9	8

and of A V were of such a titer that one part of the patient's plasma in 100 parts of normal blood prolonged very appreciably the clotting time of the latter. In the case of F Y, one part of the patient's plasma in ten parts of normal blood was required to produce an unequivocal delay in clotting.

Nature of the anticoagulants

Several studies were carried out in an attempt to identify the clotting inhibitors in the blood of these patients. In an effort to demonstrate

a possible relationship of the anticoagulants to heparin, the effect of toluidine blue and of protamine on the clotting time was observed. Serial concentrations of toluidine blue and of protamine, which were very effective in shortening the clotting time of heparinized normal blood, were used. However, there was no significant effect of these agents on the clotting time of blood from these patients.

Assays of the plasma for proteolytic enzyme and for proteolytic enzyme inhibitor were made by Dr. Oscar D. Ratnoff and Dr. David Grob. Essentially normal values of both were obtained in each instance.

In the case of F. Y., the hemophilic, precipitin tests were set up with the patient's serum against normal plasma and against a solution of Fraction I of Cohn (containing antihemophilic globulin). No precipitins could be demonstrated.

Platelet-free plasma from each of the patients was dialyzed against 0.85% sodium chloride solution without loss of anticoagulant activity. In each case the anticoagulant withstood heat at 65°C. for 5 minutes.

Antithrombic activity of the anticoagulants

Tests were performed to determine the phase of blood coagulation in which the anticoagulants exerted their effects. The prothrombin times of patients J. H. and F. Y. were within the normal range and it seemed unlikely that their anticoagulants could be antithrombic. However, the prothrombin time of A. V. was moderately prolonged.

Oxalated plasma from each patient was mixed with a solution of thrombin. The clotting times obtained were compared with the clotting times of a series of normal plasmas similarly treated. Table 2 shows the results of this test.

The data reveal that none of the plasmas showed an increase in antithrombic activity. Nevertheless, it seemed probable that the prolonged prothrombin time of patient A. V. might be accounted for by the presence in his blood of an anticoagulant rather than by a deficiency of prothrombin. Evidence for this was provided by another type of examination.

Oxalated plasma from each of the patients was treated with barium sulfate to remove all of the prothrombin. The prothrombin-free plasmas thus obtained were used as diluents for a normal oxalated

plasma, one part of the normal plasma being added to four parts of prothrombin-free plasma in each instance. The actual prothrombin concentration in each of these mixtures was 20% of the normal, since all of the prothrombin present was supplied by the portion of normal plasma. As a control, a portion of the normal plasma used in the test

TABLE 2

Clotting time at 37°C of 0.1 cc oxalated plasma on addition of 0.1 cc bovine thrombin solution

Each figure represents the mean of several determinations. The tests on the 3 patients were done on different days with different thrombin solutions and therefore cannot be compared with each other but only with the accompanying control value. Several normal control plasmas were used in each instance and the results averaged.

	J H	NORMAL CONTROLS	A V	NORMAL CONTROLS	F Y	NORMAL CONTROLS
Thrombin time (seconds)	27	30	34	38	34	36

TABLE 3

The prothrombin time of a normal plasma diluted to 20% with normal prothrombin-free plasma compared with an equal concentration of the same normal plasma diluted with prothrombin-free plasma from the patient

The tests on the 3 patients were done on different days with different normal plasmas and cannot be compared with each other but only with the accompanying control.

	SOURCE OF PROTHROMBIN FREE PLASMA					
	J H	Normal Control	A V	Normal Control	F Y	Normal Control
Prothrombin time of normal plasma diluted to 20% with pro- thrombin free plasma (seconds)	41	40	50	33	46	48

was also treated with barium sulfate and was similarly employed as a diluent for the untreated normal plasma. The results of the prothrombin time determinations on these plasma mixtures are shown in Table 3.

When the prothrombin-free plasma of patient J H or of F Y was used as a diluent for a normal plasma, essentially the same prothrombin

time was obtained as when a normal prothrombin-free plasma was used as a diluent. However, prothrombin-free plasma from patient A V definitely prolonged the prothrombin time of the normal plasma. It seems evident that the prolonged prothrombin time of patient A V was the result of the presence in his blood of a clotting inhibitor.

Antithromboplastic activity of the anticoagulants

The antithromboplastic activity of plasma was estimated by determination of the clotting time of platelet-free plasma on addition of a suspension of acetone-extracted rabbit brain thromboplastin. The

TABLE 4

Clotting time in seconds (37°C) of platelet-free plasma (silicone technic, no anticoagulant added) on addition of an emulsion of acetone-extracted rabbit brain thromboplastin

Different thromboplastin preparations were used in the 2 tests which are therefore not comparable. In test II the plasma of an ordinary hemophilic (Hem) was used for comparison instead of normal plasma.

TEST	SOURCE OF PLATELET FREE PLASMA	THROMBOPLASTIN DILUTIONS					
		Undilute	1 10	1 50	1 100	1 200	1 400
I	Normal	15	19	56	108	240	320
	A V	25	71	>600	—	—	—
	F Y	15	21	66	170	307	419
II	Hem	15	27	58	83	160	335
	J H	15	23	45	56	79	138
	A V	23	77	196	250	525	—

plasmas used in the test were obtained by the silicone technic in the same manner as for the anticoagulant assay previously described. Although the platelet-free plasma from each of the patients could be kept indefinitely in glass tubes without evidence of clotting, coagulation occurred promptly on addition of thromboplastin. Serial dilutions of the thromboplastic preparation were used in the test. Table 4 shows the results of two of these assays.

The response to thromboplastin of the platelet-free plasmas of J H and F Y appeared to be essentially normal. However, the clotting times of the plasma of A V were considerably prolonged. The plasma of A V has already been shown to contain a clotting inhibitor which

delays the conversion of prothrombin to thrombin. Whether this inhibitor is actually antithromboplastic, or whether in some other way it inhibits activation of prothrombin, could not be established. However, the results indicate that with decreasing concentrations of thromboplastin the effect of the clotting inhibitor was much more evident. When oxalated plasma from patient A V was incubated with thromboplastin before recalcifying, there was no evidence of inactivation of thromboplastin.

DISCUSSION

A number of case reports have appeared in the literature in which atypical hemorrhagic diatheses have been found to be associated with prolongation of the coagulation time. In most of these cases the cause of the delay in clotting was not determined. For example, Madison and Quick (7) in a recent paper describe a case and mention several others in which a hemophilia-like disease was observed in the female. In these cases no anticoagulant assay was reported. It seems not improbable that in at least some of these patients a circulating anticoagulant may have been the cause of the coagulation defect.

In 1940, Lozner et al (6) reported the case of a 61 year old mulatto with a hemorrhagic disorder associated with the presence of a circulating anticoagulant. The patient was found to have a positive serologic test for syphilis and glandular tuberculosis. The source of the anticoagulant was not determined. The anticoagulant was relatively thermostable and non-dialyzable. Its effect was not altered by protamine and therefore it did not appear to be heparin. The prothrombin time was reported to be normal, so that it seems likely that the anticoagulant must have interfered with the first stage of coagulation.

A circulating anticoagulant developing in the plasma of hemophiliacs following repeated transfusions has been described by Lawrence and his associates (3, 5) and by Munro (8, 9, 10). Three such cases have now been reported. Study of these cases showed that the anticoagulant was associated with the gamma globulins. Craddock and Lawrence (3) were able to demonstrate in the sera of their patients precipitins against normal plasma and against Cohn's Fraction I containing the antihemophilic globulin. They believe that the anticoagulant in the hemophiliac is an antibody against the antihemophilic globulin.

Chargaff and West (2) reported the case of a woman with a hemophilia-like disorder in whom the prolonged clotting time was caused by a circulating anticoagulant. The anticoagulant was not inhibited by protamine. It was not antithrombic, but its effect was overcome by tissue extract thromboplastic protein. No cause for its appearance in the blood was found.

A hemorrhagic disorder in a 39 year old female was described by Fantl and Nance (4). The patient was shown to have a circulating anticoagulant which appeared to be antithromboplastic for human brain thromboplastin but not for rabbit brain. There was no antithrombic activity. The bleeding tendency in this patient appeared several months after a normal pregnancy.

Castex (1) mentioned briefly 5 cases of "pseudohemophilia" with prolonged coagulation time in which the clotting time was shortened in vivo and in vitro by protamine. He attributed the disorder to the presence of heparin in the blood. The clinical situations were not described.

The anticoagulants which we have described appear to be different in each of the three cases. If the thesis of Craddock and Lawrence is accepted, the clot inhibitor in the hemophiliac is of antibody nature, developing as a result of therapeutic administration of a protein present in normal blood but foreign to the hemophiliac. We were unable to demonstrate precipitins against antihemophilic globulin in our hemophilic patient, but a relationship between previous transfusions and the production of the anticoagulant seems not unlikely. Patient J. H., whose anticoagulant resembled that of the hemophiliac in its mode of action, had not been transfused before his coagulation disorder occurred. His anticoagulant must have been produced by some other mechanism. The clotting inhibitor of A. V. was very different from the others in its mode of action. The anticoagulant in each patient was relatively heat stable, non-dialyzable and probably protein in nature.

The anticoagulants of patients J. H. and F. Y. did not appear to be antithrombic nor antithromboplastic. Their action, therefore, must precede the liberation of active thromboplastin in the blood. It seems probable that they exert their effect by preventing the conversion of a thromboplastin precursor to its active state. On the other hand, the clotting inhibitor in the plasma of A. V. definitely delayed the con-

version of prothrombin to thrombin even in the presence of an excess of active thromboplastin

The discovery of cases such as we have reported emphasizes the importance of carrying out anticoagulant assays on the blood of all patients with prolonged coagulation time. It seems probable that these cases are not as rare as a survey of the literature would lead one to believe. No doubt many of the reported cases of hemophilia-like disorders could have been shown to be of this type. These cases present a serious problem with regard to therapy. The anticoagulants are so potent that their effects obviously could not be overcome even by massive transfusions. We know of nothing which will serve as an antidote to their actions.

SUMMARY AND CONCLUSIONS

1 Three cases are reported in which a hemorrhagic diathesis with prolonged clotting time has been found to be associated with the presence of an anticoagulant in the blood

2 Studies on the nature and mode of action of these anticoagulants are described

3 The importance of performing anticoagulant assays on the blood of patients with prolonged clotting times is emphasized

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THE CLINICAL MANIFESTATIONS OF THE SEVERE FORM OF DIPHTHERIA

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I INTRODUCTORY REMARKS

It is well known that diphtheria is caused by the growth of virulent *C. diphtheriae* organisms in various tissues of a susceptible host, resulting in the elaboration and absorption of a soluble exotoxin capable of producing serious damage to some of the body's most vital cells. The primary infection in the majority of cases occurs in the respiratory tract, though lesions in the skin are not uncommon and other sites are occasionally involved. This primary infection is an intense inflammatory reaction producing edema of the surrounding tissues and usually a tough, membranous type of superficial exudate. This *bacterial* infection tends to be a localized one, and though the whole respiratory tract may often be involved from the nares to the bronchioles, invasion of the deeper tissues by the organisms with suppuration or spread into the lymphatics or blood stream almost never occurs. The *toxin*, on the other hand, is rapidly absorbed and is responsible for the systemic symptoms characteristic of the disease. Certain types of cells—particularly those of the myocardium, the nervous system and the adrenals—seem to be more susceptible to its action than most of the other tissues of the host. The therapeutic approach to the problem, therefore, is concerned with relief of obstructive symptoms caused by this peculiar type of inflammation in the respiratory tract, and always calls for neutralization of the circulating toxin as quickly as possible, before it is fixed by the susceptible cells.

Because of the extensive programs that have been instituted throughout the whole country, and especially in the large cities, for control of diphtheria by immunization with toxoid, there is a dangerous tendency for the medical profession as a whole to feel safe regarding this in-

fection. However, diphtheria still presents a problem of great importance to the general medical profession, and several reasons may be given here in support of this statement.

1 There has been an increase in prevalence of diphtheria throughout the United States in the past few years (1). This certainly has been true in Baltimore, where in 1944 there were 227 cases with 13 deaths, in 1945, 352 cases with 19 deaths, and in 1946, 419 cases with 20 deaths. This is in contrast to the situation in 1943 when the incidence was 109 cases with 3 deaths and in 1942 when 74 cases were reported, with no deaths. In 1947 the incidence dropped again, there being 143 cases with 4 deaths (2). In considering these outbreaks and the common occurrence of the severe and even malignant form of diphtheria, one wonders at once whether or not a new type of corynebacterium may be responsible. However, no evidence has been obtained to indicate that this can be related either to strain difference or to an increase in virulence of the organisms. There is a definite possibility, however, that such outbreaks may be due, at least to some extent, to the large influx of war workers from rural areas, such groups being, presumably, more susceptible to the infection, due either to lack of immunization or lack of previous contact with the organisms.

2 A second point that deserves emphasis is that diphtheria is too often considered a problem primarily for pediatricians. However, one purpose of the present study is to show that such an attitude is neither a logical nor a safe one, for there have been many instances of the infection in young adults and even in patients in the older age groups. There actually seems to be a tendency to a rising age incidence, this is fairly definite in the Baltimore statistics and there seems to be a similar trend in other parts of the country. These figures likewise must have been influenced to some extent by the crowding of non-immune war workers into the large cities. This point of age incidence is further emphasized when we consider the common occurrence of diphtheria outbreaks in the service personnel during the years of the War, such outbreaks having been encountered in all parts of the world.

3 It has likewise been noted in the recent epidemics in Baltimore that many of the patients gave a definite history of previous immunization (3). This same phenomenon was reported in 1946 from a Canadian Province (4) and also in a "highly immunized community" in

Great Britain in 1947 (5) Consequently, one cannot rely on a history of toxoid administration to rule out the diagnosis of diphtheria, nor can one always rely on a history of a previous attack of diphtheria to rule out the possibility as there are occasional instances in which two definite attacks have occurred in the same individual

4 There is another fact that has been thoroughly established but often lost sight of, namely that most cases of diphtheria can be cured if recognized early and *treated immediately* with adequate amounts of antitoxin The great importance of the time factor does not seem to be adequately appreciated The diphtheria toxin is comparable in many ways to that of tetanus, and also to many viruses If it is not inactivated by antibodies it is taken up by susceptible cells and evidently fixed by them in such a way as to be protected from the antibodies Its damaging effect on these cells may not be apparent at once but may appear a few days or weeks later as some of the serious delayed manifestations of the infection A good example of the way in which diphtheria toxin may be fixed rapidly by cells is shown in the virulence test in which the reaction is carried out in the skin of the rabbit In this test a small amount of the culture filtrate is injected into the shaved abdominal skin of the rabbit, and 5 hours later a large dose of antitoxin is given intravenously In a positive test, in spite of the presence of a great excess of antibodies in the circulating blood, a wide area of skin shows signs of an acute inflammation, which reaches its height long after the antitoxin has been injected In other words, though the spread of the toxin may be limited in this manner and its systemic effects prevented, the local *cellular* reaction continues, apparently uninfluenced by the antitoxin Consequently, if we are justified in comparing this reaction to the natural infection, we have an additional argument for the possible life-saving effect of diagnosing and treating diphtheria in its earliest stages, before damage to vital cells has become too great or irreversible

II THE CLINICAL PICTURE OF DIPHTHERIA NOTED IN A SERIES OF 30 CASES RECENTLY OBSERVED

The object of the present study was to point out the clinical and pathologic features of the more severe forms of diphtheria by an analysis of a group of 30 cases which have recently come under observation

Five of these patients were treated at The Johns Hopkins Hospital and 25 at The Sydenham Hospital for Infectious Diseases. They were all seen in the past few years, and except for 3 of those at The Johns Hopkins Hospital they were seen in 1945-1947. The selection of cases was based on their ability to demonstrate the salient features of the severe form of the acute illness which we wished to point out. There were no children in this group under the age of 3 years and many were older children or adults. In our selection we tended to pick out a group of patients who were older than the average of those admitted to The Sydenham Hospital. Consequently, though there has been an increase in the age level of patients with diphtheria in Baltimore in the past few years, our figures in this small series are not meant to emphasize that point. Some of the cases were problems in diagnosis, most of them showed the dangerous complications or manifestations that may occur early or late, and a few served to illustrate the "malignant" type of infection. The chief points of interest are illustrated in Table I.

From a consideration of the data shown in the table, and from more complete analysis of the patients' records, the clinical picture of diphtheria as demonstrated by this group of cases may be analyzed as follows:

(A) *History and Symptoms* Aside from a story of exposure to a known or suspected case of diphtheria, there is little of real diagnostic importance in the patients' symptoms that will differentiate this from other acute infections of the upper respiratory tract. The initial symptoms are usually sore throat and rather marked prostration, while the temperature is only moderately elevated. Vomiting is a common occurrence, particularly in children, but probably not more so than in other acute infections. Following this there are frequently symptoms of partial obstruction to the nares, pharynx or larynx depending on the location of the infection. A sanguineous discharge from the nose is very suggestive of diphtheria, though its incidence is not high. The diagnosis is seldom suspected during the first 2 or 3 days, and the patients are usually given symptomatic treatment or one of the sulfonamides. As was mentioned previously, a good proportion of the cases in these recent outbreaks has been in patients giving a history of immunization with toxoid—12 out of 30 being in that category in our

series This problem deserves further analysis and study in order to determine whether or not enough doses of toxoid had been given, whether the standard toxoid is maximally antigenic, etc

Another point which serves to emphasize the fact that the history (and even the preliminary examination of the patient) does not lead to an early diagnosis is shown by the lapse of time before antitoxin is given In these 30 patients the duration of the symptoms prior to diagnosis and treatment varied from 2 to 17 days, with an average of 4.7 days The chief reason, however, for this failure to make an early diagnosis in diphtheria is not so much the lack of specificity of the symptoms and signs as it is the failure of the physician to consider this as a prominent possibility in all patients with acute infections of the upper respiratory tract

(B) *Physical Findings* The patient with diphtheria is apt to appear more ill and prostrated than one would expect from the temperature elevation and there is often tachycardia which is out of proportion to the fever, in comparison with other acute respiratory infections A bloody nasal discharge is a very important finding from a diagnostic standpoint—as mentioned earlier The *local lesion*—whether it be in the nose, throat or larynx—is usually a tough, membranous type of exudate attached firmly to the tissues and tending to be confluent rather than spotty or punctate The borders of the “membrane” are usually fairly sharply demarcated and the surrounding tissues—particularly those in the pharynx—show varying degrees of edema and redness This edema at times tends to be so marked as to make it nearly impossible for one to get a good view of the posterior pharyngeal wall and the exudate itself may be entirely hidden It is in this type of case that the diagnosis of diphtheria is frequently overlooked and precious time may be lost in waiting for the culture reports The most typical local lesions are those in which there is a membrane in the nares or the larynx, or on the posterior pharyngeal wall and extending up over the soft palate and uvula In such cases a positive diagnosis can be made at once and antitoxin given immediately

Cervical adenitis of greater or lesser degree is present in nearly all patients, and in the tonsillar or pharyngeal types it often becomes very marked Frequently in the patients who are severely ill the adenitis is accompanied by a marked edema that is brawny in character, tender

TABLE I
Summary of Clinical, Laboratory, and Other Data in 30 Cases of *D. philipina*

Summary of Clinical, Laboratory, and Other Data in 30 Cases of D.P.										
NAME AND NUMBER	AGE	PREVIOUS IMMUNIZA- TION (AGE)	VIRULENCE TESTS ¹	OTHER BACTERIA (1)	DURATION (IN DAYS) PRIOR TO ANTITOXIN ACTION)	N P N (HIGH- EST DE- TERMIN ATION)	OUTCOME (2)	CHIEF CLINICAL AND PATHOLOGICAL FINDINGS		Significant Autopsy Findings (exclusive of local lesions)
								Clinical Features		
								Cardiac ab- normalities (3)	Other manifestations or complications	
1 H A, 278665 ¹	14	?	+	β -strep	4	88	R	+	Laryngeal obstruc- tion Bullneck Acute Nephritis (4)	
2 C O, 287990	51	?	-	-	4		R	+	Laryngeal obstruc- tion Bullneck "Pentonsillar ab- scess" (5)	
3 A F, 34685	10 (6 mos)	+	-	β -strep	2		D (13 days)	+	Bullneck Laryngeal obstruc- tion Diabetes mellitus (severe) Acute Nephritis (4)	
4 J R, 343667	17	0	+		4	150			Palatal Paralysis Peripheral Neu- ritis Circulatory lapse geal obstruction Bullneck	Adrenal Cortical damage (6) Pe- techial hemor- rhages in myo- cardium
5 I G, 355002	34	?	+	Staph aureus	5	38	R	++		
6 J G, 380105	35	?	+	-	3	57	D (2 days)	++		

7 K G, 33999	6 +	+	+	+	α strep	9	70	D (34 days)	++	Laryngeal obstruction Atelectasis Diaphragmatic paralysis	Myocarditis (lesions showing healing & necrosis) Lobular pneumonia Laryngeal obstruction Atelectasis (Slight)
8 R H, 33975	27	0	+	+	-	2	D (4 hrs)	0	++	Laryngeal obstruction Bullneck	
9 A G, 33874	59	0	+	+	β strep α strep α-strep	4	D (1 day) R	++	++	Arteriosclerotic Heart Disease Palatal and ocular Paralysis Circulatory Collapse	
10 C K, 33773	11	+	+	+	α strep	3	D (6 days)	++	++	Laryngeal obstruction Bullneck	
11 G K, 34174	4	0	-	-	α strep	6	D (6 days)	++	++	Arteriosclerotic Heart Disease Palatal and ocular Paralysis Circulatory Collapse	
12 J G, 34036	7	+	+	+	-	4	D (6 days)	+++	+++	Laryngeal obstruction Bullneck	
13 J K, 33958	28	0	+	+	β strep staph	17	D (65 days)	+++	+++	Circulatory Collapse Bullneck Anemia	
14 R L, 33658	18	?	+	+	α strep β strep Pneumo	2	D (10 days)	+++	+++	Laryngeal obstruction Polypneumonia "Pneumonic Abscess" (5)	Myocarditis (extensive and healed) Lobular pneumonia
15 M R, 33840	12	+	-	+	α strep β strep	5	D (9 days)	+++	+++	Circulatory Collapse Tracheobronchial obstruction Old Polymyositis Circulatory Collapse Palatal paralysis Bullneck	Myocarditis Lobular pneumonia Adrenal Cortical damage (slight) Myocarditis Adrenal cortical damage

TABLE I—Continued

NAME AND NUMBER	AGE	PREVIOUS IMMUNIZATION (AGE)	VIRULENCE TESTS ²	OTHER BACTERIA (1)	DURATION (IN DAYS) PRIOR TO ANTITOXIN	N P N (HIGHEST DE TERMINATION)	OUTCOME (2)	CHIEF CLINICAL AND PATHOLOGICAL FINDINGS		
								Clinical Features	Cardiac abnormalities (3)	Significant Autopsy Findings (exclusive of local lesions)
16 J K, 34464	11	0	+	α-strep β-strep (few)	2		D (5 days)	Circulatory collapse	+++	
17 W B, 34352	6	0	—	α strep β-strep	3		D (1 day)	neck Bullneck	+	
18 H S, 33678	4	+	+	—	5	64	D (1 day)	Laryngeal obstruction	++++	
19 R B, 33691	3	+	+	α-strep β-strep	5	103	D (2 days)	Tracheo-bronchial obstruction	+	Purulent Bronchitis Lobular Pneumonia Atel-ectasis
20 R R, 34746	11	+	+	β-strep	4		R	Bullneck	+++	
21 D K, 33776	6	+	+	α-strep	5	104	D (4 days)	Circulatory collapse	+++ (With failure)	Myocarditis Adrenal cortical damage Lobular Pneumonia
22 P W, 34492	9	0	+	β strep	5	206	R	Broncho-pneumonia & Tracheal obstruction	+	

23 T L, 34763	7	+	—	Pneumo VI β-strep α-strep β strep	4	154	D (12 days)	+++	Circulatory Col- lapse Bull- neck	
24 K W, 35040	17	(2 mos ago) 0	+		3	60	D (11 days)	+++ (With failure)	Circulatory Col- lapse Pharyn- geal Paralysis Bullneck	Myocarditis Ad- renal cortical damage Atro- phy of one ad- renal
25 G K, 35154	6	?	+	α strep	2	86	D (3 days)	+++	Circulatory Col- lapse	Myocarditis Ad- renal cortical damage Lobu- lar Pneumonia
26 B S, 33554	17	+	+	α strep β-strep	4	33	D (3 days)	0	Circulatory Col- lapse Laryn- geal obstruction Cerebral anoxia Bullneck	Adrenal cortical damage (slight) Focal hemor- rhages in heart and lungs
27 F H, 35226	70	?	—	E Coli	6	78	D (17 days)	++ (With failure)	Bronchopneu- monia Laryn- geal obstruction Palatal Paraly- sis Nephritis?	General and co- nary Arterio- sclerosis Focal myocardial scar- ing Lobular Pneumonia Spotty necrosis and hyaline droplets in renal tubular epithe- lium
28 A S, 34636	5	+	+	—	3		R	+	Laryngeal obstruc- tion Pharyn- geal Paralysis Paralysis of legs	

TABLE I—*Concluded*

NAME AND NUMBER	AGE	PREVIOUS IMMUNIZA- TION (AGE)	VIRULENCE TESTS ²	OTHER BACTERIA (1)	DURATION (IN DAYS) PRIOR TO ANTITOXIN	N P N (HIGH- EST DE- TERMINA- TION)	OUTCOME (2)	CHIEF CLINICAL AND PATHOLOGICAL FINDINGS		
								Clinical Features		Significant Autopsy Findings (exclusive of local lesions)
								Cardiac ab- normalities (3)	Other manifestations or complications	
29 E M, 34094	6	0		—	14		D (12 hrs)	+++	Cerebral thrombo- sis?	Myocarditis Thrombi in auricles and left ventricle
30 J W, 34743	5	0	+	α -strep	3		R	++	Palatal Paralysis Bullneck	
Totals		12 positive	6 negative	β -strep 14 cases	Average 4 7 days		D 21 R 9	27	Laryngeal or Tracheal ob- struction 15 Bullneck 18 Circulatory Col- lapse 11 Paralyses 9	Myocarditis 9 Adrenal cortical damage— Marked 5 Slight 2 Pneumonia 7

¹ The six-figure numbers represent Johns Hopkins Hospital histories, the others are from Sydenham Hospital

² Culture for *C. diphtheriae* was positive in each case + = positive virulence tests, — = negative tests

(1) Heavy growth unless otherwise stated — = no significant growth

(2) R = recovered, D = Died (in days after admission)

(3) Rough estimation of degree of abnormality as determined clinically and by Ekg

(4) Diagnosis probable but not proved

(5) Primary diagnosis, which was incorrect

(6) Includes both necroses and "tubular degeneration" of adrenal cortical cells

to touch, and which obliterates the normal curves between the mandible and the clavicle. This results in the so-called "bull-neck" appearance. This condition has been encountered so frequently during the recent epidemics, and has been associated so regularly with the most severe types of infection, that the term "bull-neck" diphtheria is often used to describe a particularly malignant variety. One wonders whether in these cases there may not be some strain difference of the infecting microorganism, which is not determined by the usual biological tests or whether other organisms may play a secondary role. Further studies should be done in hopes of clarifying these points, but routine cultures so far have not revealed secondary invaders that could be considered specific in producing this reaction. In the present series the bull-neck swelling was a prominent feature in 18 cases (60%).

Laryngeal or tracheo-bronchial obstruction was encountered in 15 (50%) of the patients and was nearly always relieved by prompt tracheotomy and suction. The suction could usually be accomplished successfully by rubber catheters but repeated bronchoscopic aspiration is often necessary. These patients show varying degrees of obstruction, with difficult wheezing respirations, cyanosis, and retraction of the interspaces or of the whole chest wall with inspiration. Signs of atelectasis or pneumonia also may be present. After tracheotomy pieces of membrane may be aspirated, and at times whole casts of the trachea or of the tracheo-bronchial tree can be obtained. Such procedures result in dramatic improvement in the patient's condition, but may need to be repeated many times. An indication of the success of this therapy is shown by the fact that atelectasis seemed to be an important contributing cause of death in only one of this group of patients, and in one other it was felt that tracheotomy may have been delayed too long, thus resulting in damage to the brain from anoxia. None of the deaths, however, could have been attributed entirely to laryngeal obstruction.

The Heart The cardiac manifestations of diphtheria will not be discussed in any detail in this report as that aspect of the disease will be dealt with specifically in a study of a large group of patients from The Sydenham Hospital (6). Most patients who suffer from the severe form of diphtheria show evidence of myocardial involvement, and a high proportion of the deaths are due directly to this "complication."

In our group, 27 cases showed evidence of *myocardial damage*. This is usually determined—particularly in the sickest patients—on clinical grounds, but in others only electrocardiographically. The pathologic findings will be mentioned later. The myocardial damage may occur during any stage of the infection, in some it occurs early and as part of an overwhelming toxemia, in most it is found during the height of the disease, and in others it appears late, and the symptoms may develop suddenly when the patient seems well on the way to recovery. Many times it is accompanied by sudden circulatory collapse, this, however, is not always associated with demonstrable myocardial changes pathologically, as will be brought out later. The most reliable *clinical signs* of diphtheritic myocarditis are a soft or weak first heart sound, particularly if a definite change has been noted in this sound from previous observation, an increase in heart rate, a fall in blood pressure, and in some cases enlargement of the liver. This latter finding is most commonly noted in young children. Arrhythmias of various types may be noted, extrasystoles and complete heart block being apparent from clinical grounds, and tachycardias such as ventricular tachycardia and auricular flutter usually being diagnosed by the electrocardiogram. Signs of congestive failure may occur in a few patients—if the patients survive long enough—but this is not a common finding. During the acute phase of the myocarditis in the patients studied recently at The Sydenham Hospital there was frequently found an increase in circulation time and sometimes a moderate increase in venous pressure. Further studies are being carried out along these lines, but one of us (S C), has observed enough patients with these findings to feel that they offer methods by which the diagnosis of myocardial weakness may be determined early and a means of following the efficacy of treatment.

The *electrocardiographic* changes are varied and not specific for diphtheria, but alterations from the normal tracing are observed so commonly—even in the milder cases—that the electrocardiogram offers us a useful test for diagnosis and prognosis of this complication. In the cases we are discussing the changes varied from the milder type, in which there was a slight alteration in the T-waves or increase in conduction time, to the most severe types such as complete heart block. The most significant findings in this group—with most patients show-

ing more than one abnormality—may be summarized roughly as follows. Lowering or inversion of T-waves was noted in 14 instances, depression of the S-T segments in 11, incomplete heart-block (including slight prolongation of the P-R interval) in 5, right axis deviation 5 times, and lowered voltage 5 times. Complete auriculo-ventricular block occurred 12 times and intraventricular block 9 times. Both of these were, of course, interpreted as evidence of advanced myocardial damage and as very grave prognostic signs, in most cases being found shortly before death. Ventricular tachycardia was diagnosed three times, auricular flutter twice, and auricular fibrillation once. In most of these patients, though it was felt very likely from clinical findings alone that myocarditis was present, the electrocardiograms were of great importance, not only in determining the degree of myocardial damage but also in following its trend from day to day or even from hour to hour.

Circulatory Collapse It is well recognized that shock is a common occurrence in many types of acute infection, and this is particularly true of diphtheria. Rich (7) observed necrosis and “tubular degeneration” in the fascicular layer of the adrenals in many patients dying of severe acute infections including diphtheria, and speculated on its probable importance in producing circulatory collapse. There were 5 instances in the present series in which this pathological finding was noted in patients who developed sudden circulatory failure just before death, and it was felt that it may have played an important role in the fatal outcome. In one of these there was no microscopic evidence of myocarditis. However, the exact importance of this adrenal lesion cannot be definitely stated at the present time, and attempts at replacement therapy with adrenal cortical substance so far have not proved effectual, though the dosage employed may not have been adequate. Perhaps further studies on a larger series of cases will throw more light on this particular aspect of the problem.

The Nervous System Paralysis of various cranial or peripheral nerves is a common accompaniment of diphtheria, the former occurring most often in the acute phase or slightly later, and the latter usually being delayed—sometimes as late as six weeks or longer after the acute attack. This late type of nerve lesion, which apparently has no analogue in any of the other infections in which neurotropic toxins or viruses are involved, must be due to early fixation of the toxin in the nerve cells and

then to very gradual changes in those cells, often taking weeks to develop to the clinical stage of peripheral neuritis. The recovery of the paralyzed muscles is slow, but usually complete. The earlier paralyses, which are most apt to involve the palatal muscles, are more serious complications because they usually occur when the patient is most acutely ill and make the problems of feeding and respiratory exchange more difficult during this critical period of the infection. This early type of paralysis is due to muscular damage, caused by local diffusion of the toxin, while a later type is a true nerve palsy and may come on three weeks or more after the acute onset.

As shown in the table, paralyses of one type or another were encountered in 9 patients in our group. In these cases there were 6 instances of palatal or pharyngeal paralysis, one each of ocular and diaphragmatic paralysis, and 4 instances of polyneuritis. Whereas all of these paralyses could be considered serious manifestations of the disease, the most dangerous ones were the palatal lesions, which occurred at the height of the infection in all except one patient and always in association with other complicating factors.

The Kidneys While it is very common to find a moderate degree of albuminuria at one time or another in severe cases of diphtheria, a diagnosis of *nephritis* does not seem justified unless there are repeated findings of heavy albuminuria—and often casts as well—in association with elevation of the non protein nitrogen in the blood. In 6 of our patients the diagnosis of nephritis was made on clinical grounds, but in none was it proved pathologically, as two of them recovered, no autopsy was done in another, and the other 3 showed only cloudy swelling, hydropic changes, and in one necroses and hyaline droplets in the renal tubular epithelium. Determinations of non protein nitrogen in the blood were not made in every case, but the table shows that it is frequently elevated considerably even without definite evidence of nephritis. This must be due in part, at least, to temporary renal insufficiency in association with myocarditis and impaired renal circulation, but other factors may play a role, and preliminary determinations have shown that the electrolyte pattern may be very much altered in the patients who are seriously ill with diphtheria. Such findings may be related to damage to the heart or the adrenals rather than to a true nephritis.

(C) *Laboratory Findings Leucocytosis* The white blood cell count is usually stated to be only moderately elevated in diphtheria, but most of our cases showed a rather marked leucocytosis. In this series of patients the highest counts varied from 7,500 to 56,000, with an average of 20,600 per cubic millimeter. Many of the high values may have been due to the fact that other bacterial throat infections were frequently superimposed on the diphtheria—as shown by the culture reports. No definite correlation was noted between the severity of the diphtheria and the height of the white blood cell count.

Bacteriological Studies Cultures from the throat, nose or trachea were positive in every case for *C. diphtheriae* and in some instances the organism was grown from each of these sites. The tendency for the organisms to remain localized rather than to penetrate more deeply into the tissues or to invade the lymphatics or blood stream is illustrated by the fact that, with one exception, these bacteria were never isolated from other tissues or from the blood. In the one exception, (patient T L), a positive culture was obtained from the heart's blood just after death. This strain of *C. diphtheriae* was reported as being "non-virulent" just as the throat culture had been in this typical and fatal case of diphtheria. It is probable that this represents a terminal invasion of the blood stream, but it is possible that positive blood cultures might be obtained more often in the patients who are most seriously ill. Further indication that the diphtheria bacillus may, in an exceptional instance, invade the blood stream is afforded by a recent case report of a patient with bacterial endocarditis that was shown to be due to *C. diphtheriae* (8).

It is also of interest to note that in our series the routine *virulence tests* resulted in reports of "avirulent" organisms in 6 of the cases. In one patient the strain was found avirulent in one laboratory and virulent in the other, and in another the organism recovered from the trachea was found virulent and that from the nose avirulent. In none of these patients was there any question regarding the diagnosis of diphtheria. The methods employed in all these tests were the standard guinea-pig tests, which are done at times with the mixed culture, or more accurately with a pure culture. Ordinarily these are considered reliable tests for virulence, but it is obvious from this small series that they are not always accurate. Possibly other types of reactions, either

employing the rabbits' skin or the chicken in the experiment, may prove to be more reliable, or some other animal may be found in which the diphtheria toxin produces effects more closely parallel to its effects in the human being

Other Bacteria Various organisms other than diphtheria bacilli were isolated in the routine cultures of the nose, throat or trachea from most of the patients (as shown in the table) In most cases a heavy growth was obtained, making it appear that they may have been of some clinical significance in increasing the severity of the infection This seems more likely in the case of *beta-hemolytic streptococci* than the others, this organism being recovered from 14 of the patients It is because of the frequency of such cultural findings that antibiotic or chemotherapy is justified in hopes of modifying the infection, rather than for its effect on the diphtheria itself

III DIFFERENTIAL DIAGNOSIS

A few of the more common acute infections that frequently cause difficulty in diagnosis will be mentioned briefly

(1) Acute tonsillitis or pharyngitis The majority of these infections are caused by *beta-hemolytic streptococci*, and at times are very difficult to differentiate from pharyngeal diphtheria If the pharyngeal lesion contains a confluent, membranous type of exudate, the diagnosis of diphtheria is very likely as the streptococcal infection usually results in a more patchy exudate which can be wiped off with a throat swab The diphtheritic membrane is tough in consistency and is apt to leave a bleeding surface when scraped off The patient with a streptococcal infection is apt to have a higher temperature than the one with diphtheria, and yet the latter usually looks sicker and is more apt to show signs of myocarditis Smears and cultures should be taken from beneath the exudate and incubated immediately on Loeffler's medium and on blood agar A positive smear for *C diphtheriae* may be obtained from the Loeffler's medium in 8-12 hours In a patient, however, in whom there is any suspicion of diphtheria, antitoxin should be given

(2) Vincent's infection In these cases the exudate is usually confluent and therefore hard to distinguish from diphtheria but the base has a tendency to ulcerate more deeply into the tissues In these pa-

tients a smear taken from beneath the exudate will be positive for the fuso-spirochetal organisms characteristic of the infection. In a doubtful case a presumptive diagnosis of diphtheria should be made and the patient treated accordingly. Likewise, it is well to bear in mind that in an occasional case both infections may occur at the same time, and the diagnosis of diphtheria may be missed when that of Vincent's angina is established.

(3) Infectious mononucleosis. It is a fairly common occurrence for patients with the "anginal" type of this disease to be diagnosed diphtheria on the basis of the appearance of the throat. However, the correct diagnosis is usually made by the finding of enlargement of glands other than the local ones, a palpable spleen, and abnormal lymphocytes in the blood smear. The heterophile agglutinins appear in the blood in significant titers after ten days to two weeks and help to settle the diagnosis. We were interested to find several examples, however, of patients in whom *both* infections were present simultaneously. One of these patients, who was seen at The Johns Hopkins Hospital, showed all the characteristic features of infectious mononucleosis, and was treated as such for 10 days before the presence of diphtheria was determined. He continued to have fever and a membranous lesion in the throat from which positive cultures of *C. diphtheriae* were obtained only after the third attempt. The diphtheritic throat infection cleared up rapidly after antitoxin was administered. Two similar cases were observed within the past two years at The Sydenham Hospital, but in them the correct diagnosis of diphtheria was made on admission and they were treated at once with antitoxin. We have not encountered any similar report of the co-existence of these two infections, but believe that more cases will be found if the possibility is considered more often.

(4) Laryngo-tracheo-bronchitis and "croup." A child with a typical attack of croup may be no problem in diagnosis, because there is often a history of previous attacks with mild respiratory infections, with the symptoms usually developing at night. Moreover, though there may be considerable respiratory difficulty due to laryngeal edema, the child usually does not have much fever and may not seem very sick. However, there is a type of non-diphtheritic laryngitis, often associated with tracheitis and bronchitis, in which the child has

high fever, and may rapidly become seriously ill due to the acute infection and to respiratory obstruction. Such infections are usually caused by beta hemolytic streptococci, pneumococci or H influenzae and may be very hard to differentiate from diphtheria. The diagnosis may be made by examination of the throat and the larynx, and by cultures. However, it is often necessary to give diphtheria antitoxin when the diagnosis remains in doubt rather than wait for the cultures. Many times these patients with laryngo-tracheo-bronchitis require tracheotomy, which with antibiotic or chemotherapy may be life saving.

(5) Peritonsillar Abscess. In some cases of pharyngeal diphtheria the inflammatory edema is intense and localized enough to cause the tissues to bulge forward in the nature of a peritonsillar abscess. Consequently many cases are so diagnosed and the suspected abscess is either incised or aspirated. This is a dangerous procedure in diphtheria, as the local situation is made worse rather than better inasmuch as no pus is released, and particularly because large amounts of toxin may be spread into the lymphatics and bloodstream in this way. Consequently, in any doubtful case, if it seems absolutely necessary to incise what seems to be a peritonsillar abscess, the patient should be given a large dose of diphtheria antitoxin first. This situation is illustrated by the case of J. K., (#13 in the table) as will be mentioned later. Another patient, a 10 year old girl (#3 A. F.) showed an extreme degree of peritonsillar swelling with bulging and there was a membrane on both tonsils. Those who first saw the patient felt that an abscess was present and that it should be incised or aspirated. It was decided, however, that the whole process could be explained on the basis of diphtheria and this decision was borne out by the fact that the throat lesions responded well to antitoxin. Similar situations occur fairly frequently in outbreaks of diphtheria, so that any case of suspected peritonsillar abscess should be examined critically from this standpoint and treated as diphtheria unless proved otherwise.

(6) Other Conditions. In some patients with diphtheritic myocarditis the condition at times is confused with *acute rheumatic fever*, and in the absence of joint pains the differentiation may present some difficulties. This is particularly true of the cases in which the myocardial changes are not severe and where there is no definite evidence of valve

lesions At such times the diagnosis rests on careful examination of the local lesions and on repeated throat cultures in suspicious cases

The early nerve lesions appearing during the height of the infection seldom present much of a diagnostic problem However, the *peripheral neuritis* which usually has a delayed onset is often hard to differentiate from other types of neuritis In these cases the primary infection may have been rather mild, and frequently the diagnosis of streptococcal pharyngitis had been made without taking cultures In such patients the correct diagnosis is only suspected in retrospect, but may be supported occasionally by the persistence of positive throat cultures and residual changes in the electrocardiograms The sequence of swallowing difficulty, loss of accommodation for near vision, then peripheral weakness—usually without paresthesias—is characteristic In many cases the spinal fluid proteins are elevated, with no or only slight increase in cells In this aspect, as well as in the character of the peripheral neuritis, diphtheritic polyneuritis may resemble the Guillian-Barré syndrome very closely

IV TREATMENT

The therapeutic problem in diphtheria involves three main categories (a) neutralization of the circulating toxin as rapidly as possible, (b) relief of obstructive symptoms and (c) maintenance of an efficient circulatory system As soon as the diagnosis of diphtheria is suspected, the patient is tested for sensitivity to horse serum, and if the tests are negative, the antitoxin is given at once If the tests are positive the antitoxin is given in small and increasing doses at intervals of approximately one-half hour until the total amount has been given, the usual precautions are, of course, observed to deal with possible serum reactions The usual method of administration is intramuscular for patients who are moderately ill, and intravenously for those whose infection is more severe In most cases the total dose is given in one injection, and though there are no accurate methods of determining the exact amount needed, the plan is to be sure that an *excess* of antibodies is gotten into the blood as quickly as possible In this group of severely ill patients the dosage varied from a low of 40,000 units to a high of 220,000 units, with an average of 120,000 Except for one patient, who received 40,000 units, the minimum dose was 80,000—even

in the case of small children. The one patient (J K #13), who was given the small dose, had been treated late because he had been diagnosed in another hospital as having a peritonsillar abscess. The diagnosis of diphtheria was therefore delayed because of this and he received specific therapy 17 days after the onset. The result in his case was death from myocarditis 65 days after admission, and the heart muscle showed extreme changes with destruction of cells, round-cell infiltration and fibrosis. In all the other patients the dosage of antitoxin was equal to or well above what is ordinarily considered adequate, though the tendency recently has been to give larger amounts. The average in this series of 120,000 units is quite high. This is the result of selection of the cases for their severity and the tendency to give larger doses to the more seriously ill patients. It is interesting to note, however, that in spite of this probable excess of antitoxin, the progress of the myocarditis in many of these patients is not obviously altered. In such cases the toxin presumably has been fixed by the cells of the myocardium early in the course of the infection. These tissues then may either go through a gradual or rapid process of cellular damage in spite of the antitoxin which almost certainly does not penetrate into the cell itself. Larger amounts of antitoxin would not help in such cases, but it is important to be certain that there is an excess of antibodies in the blood as long as toxin is being formed at the site of the local lesion. We will know more about the adequacy of such therapy when determinations of antitoxin levels in the blood have been made on a series of cases after specific treatment. These blood levels should be followed for several days in order to be sure of an adequate titer over the period of the acute infection.

The problem of dealing with obstructions in the various parts of the respiratory tract has been mentioned earlier and will only be summarized briefly. The wide experience at The Sydenham Hospital of Dr Horace Hodes, Dr Margaret Smith and others has served to emphasize the importance of *early* tracheotomy, before cerebral anoxia has developed and before the child has become too fatigued from severe and prolonged respiratory effort. Aspiration by suction—or particularly in those whose obstruction is lower down and who develop atelectasis—by bronchoscope, often produces dramatic improvement. An oxygen tent is frequently necessary, and it is important that the atmosphere

in the tent be saturated with water vapor and preferably kept at body temperature

In treating derangements of the circulatory system in diphtheria one is often faced with a very difficult problem, one which does not have a parallel in any other condition. The reason for this is that circulatory failure may develop early in the course of an overwhelming infection with clinical and electrocardiographic evidence of severe myocardial damage, it may develop late, when the patient seems well on the way to recovery and in these cases also is usually associated with cardiac abnormalities, or circulatory collapse may occur during any phase of the acute infection and at times may not be explicable on the basis of myocardial damage. This problem of therapy will not be dealt with in any detail here, but it has been found that digitalization is not only a safe therapeutic procedure when carried out carefully but one that may be of real benefit, even during the acute phase of myocarditis, and is not contraindicated by signs of severe damage to the conduction system (9). The treatment of this "shock", when fully developed, is very difficult and on the whole has been unsatisfactory in the patients who are critically ill. Oxygen is given and a cautious attempt may be made to support the circulation with intravenous fluids, and thus to tide the patient over the most acute phase of the illness. Other substances have been tried—such as fifty per cent glucose, ascorbic acid and adrenal cortical hormone—but without evidence of any real benefit. This whole subject is being reviewed more completely in the other communication (6).

Penicillin was given intramuscularly in most of these patients during the past 2 years or more. In spite of the fact that the diphtheria bacilli are sensitive to penicillin, it was not found that the diphtheria itself was altered by its use, the probable explanation being that there is no evidence of its having any effect on the toxin. The chief purpose of the penicillin therapy was an attempt to prevent or eliminate other bacterial infections, and possibly to decrease the amount of toxin produced, by a direct effect on the diphtheria bacilli themselves.

V PATHOLOGICAL FINDINGS

Autopsy data have been examined in 13 of the 21 patients who died. In 8 of these there was dilatation of the heart which was usually de-

scribed as marked. There was microscopic evidence of *myocarditis* in 9, this was often very extensive and involving all chambers of the heart, at other times of mild degree. In this respect the predominant changes varied from cellular infiltration to necrosis of myocardial fibers and finally to extensive areas of fibrosis. In 2 cases no lesions of *myocarditis* could be found, though on clinical grounds they were thought to have definite evidence of myocardial damage. In one of these patients—to be described later—there were advanced electrocardiographic abnormalities to support this opinion, and yet at autopsy the only microscopic changes noted in the myocardium were widespread petechial hemorrhages. One wonders whether it may not be true that in such cases the duration of the presumed *myocarditis* was too short to lead to changes which were visible microscopically. It is possible likewise that the petechiae may have played a role in production of the cardiac abnormalities. Lobular pneumonia was found in 4 cases, purulent bronchitis in 3 and some degree of atelectasis in 2. The local membranous lesion was observed in 7 instances and in the rest it had apparently disappeared and the local ulcerations healed. None of the patients showed a true “diphtheritic nephritis”. The *adrenals* were involved in 7 cases, and in 5 of these the lesions were quite marked. These changes consisted of necroses and “tubular degeneration” in the fascicular layer of the cortex as had been described previously by Rich (7). The lesions in these patients were likewise either found or verified by Dr. Rich. They may be significant in helping to explain the occurrence of circulatory collapse and death.

VI CASE REPORTS

The following two cases are summarized in order to emphasize some of the points that have been discussed.

Case 1 (K W #24 in the table) This patient was a white girl of 17 who was admitted to Sydenham Hospital on March 19th, 1947. She had never received diphtheria toxoid. About one month before admission she developed an upper respiratory infection which cleared up except for a cough which had persisted. Three days before admission she awoke with a sore throat, malaise, and anorexia. She was seen by her doctor who prescribed two pills every three hours. She vomited several times that day. Two days before admission she felt worse, had fever and severe sore throat. Her doctor returned and painted the throat with

some white liquid. That night the neck was noted to be swelling. On the next day the condition was much worse, she had great difficulty in swallowing and had a very painful neck. The diagnosis of diphtheria was then made, she was given 40,000 units of antitoxin and referred to The Sydenham Hospital. On admission the temperature was 101.2°, pulse 114, respirations 28, and blood pressure 120/84. She was acutely ill, had a bull-neck type of cervical swelling and was hoarse, but there was no marked respiratory distress and no cyanosis. The pharynx was extremely edematous, the tonsils being greatly swollen and covered by a grayish-white membrane which extended back over the posterior pharyngeal wall and up over the uvula. There was marked edema of the tissues of the neck with tenderness to palpation. The lungs were clear and the heart was normal in rhythm and in the character of the sounds. The urine showed a trace of albumin which two days later was 2 plus and on the 8th hospital day 4 plus. The white blood count on admission was 13,000 and on the 10th day had risen to 25,000. The throat culture was positive for virulent *C. diphtheriae* and for beta-hemolytic streptococci. An N P N on the day before death was 60 milligrams per cent.

The patient was put in a steam tent and given 60,000 units of diphtheria antitoxin intravenously and 40,000 units intramuscularly. Penicillin was given in an initial dose of 60,000 units and then 30,000 units every 3 hours. She was extremely sick the first 4 or 5 days, but then seemed to be improving. The edema of the neck cleared gradually and at the time of death was entirely gone. The membrane in the throat also gradually disappeared. The blood pressure and heart sounds were normal during the first 4 or 5 days. On the 4th day she developed pharyngeal paralysis, and because of this, fluids were given intravenously and very carefully, frequent observations of the venous pressure on these days showed a moderate elevation before fluids were given and a considerable rise immediately afterward. On the 6th day she had bigeminal cardiac rhythm and the liver was down three finger-breadths below the costal margin. Digitalization was begun at that time, using the calculated dose of digalen intramuscularly over a period of 36 hours. During the first few days the electrocardiogram had shown only minor changes suggesting myocarditis, but on the 7th day there was evidence of very marked damage with inverted T-waves, bigeminal rhythm, and bundle branch block. On the 8th day she had improved, could swallow without regurgitation, and the heart sounds were better, though the first sound was definitely soft in character. The liver was no longer enlarged and the venous pressure normal. On the 9th day the blood pressure fell to 95/75, the patient had developed a striking pallor, and the electrocardiogram still showed marked myocardial damage. On the 11th day the blood pressure was 76/64, she looked very weak and listless, and the first heart sound was extremely weak and the circulation time prolonged. On the 12th day—the day of death—the temperature rose to 101° and she complained of joint pains. On that afternoon the blood pressure became unobtainable and the apical rate was counted at 200. An electrocardiogram at this time showed ventricular tachycardia. She was given quinidine intravenously, also plasma and was

put into an oxygen tent The respirations became more and more difficult and she died one hour later

At *autopsy* it was found that all the chambers of the heart were greatly dilated and the heart muscle appeared slightly paler than normal It was of interest to find that all local traces of diphtheria had cleared up, and this was noted both grossly and microscopically Histologically there was found to be an extreme degree of myocarditis and widespread necroses of myocardial fibers, these changes were present in all chambers of the heart Old necroses were found in the renal tubular epithelium which were interpreted as possibly being due to sulfonamide therapy, (this may have been administered before her admission but only in small doses and for a short time) One adrenal was atrophic, due to the presence of an angioma which was thought to have interfered with the circulation In the other, there were necroses in the fascicular layer of the cortex The anatomical diagnosis was Diffuse myocardial necrosis and myocarditis Necroses of renal tubular epithelium Slight pulmonary edema Angioma of right adrenal with atrophy, and necroses in the cortex of the other adrenal

In summary this was a patient whose acute illness began rather gradually without seeming very serious until the third day During this time the correct diagnosis was not suspected, though a bull-neck swelling had begun by the second day Following admission her condition rapidly became worse, myocarditis developed with evidence of myocardial failure She then seemed to respond to therapy and for a while her condition was improved This was only temporary, however, for in spite of the fact that she had received antitoxin at the end of the third day of her illness, there was evidence of progression of the myocardial damage until the time of her death 11 days after admission The clinical features could be explained by the extensive and fully developed changes of myocarditis, as well as the adrenal cortical damage, which may have played an important role in the final circulatory failure

Case 2 (J G #6) This patient was a 35 year old white man who was admitted to The Johns Hopkins Hospital on March 25th, 1946, and died two days later About one week before admission he had a mild earache which cleared up after local application of some drops Two days before admission he developed a sore throat which rapidly became worse A liquid medicine was given for this without relief and then penicillin lozenges were prescribed The night before admission the sore throat was much worse and the neck became swollen This swelling was much more marked the next morning and there was difficulty in breathing He was brought to the hospital in an ambulance On admission his temperature

was 103.4°, pulse 108, respirations 18, and blood pressure 90/65. He appeared acutely and seriously ill, there was a bull-neck swelling from the jaws to the clavicles, and respiratory difficulty was quite marked, but there was no cyanosis. There was a thin yellowish, bloodstained discharge from the nares. The pharynx itself could not be seen because of enormously swollen, edematous tonsils and peritonsillar tissues, the surface of which had a clear, shiny appearance. The airway was almost completely obstructed by these swollen tissues and no exudate was seen. The patient was expectorating bloody, thin, purulent material. The whole neck was greatly swollen and was red, indurated and tender. The lungs were clear except for distant breath sounds. The heart was regular and the sounds were considered normal. Inasmuch as no exudate and no membranous lesion was seen, the condition was thought to be a streptococcal infection and he was started on penicillin, 80,000 units every 3 hours, hot saline throat irrigations, and steam inhalations. The urine on admission showed a one plus reaction for albumin and many white blood cells and cellular casts. On the second day the albumin was 3 plus and there were many cellular casts. The white blood count was 17,400 on the first day. The first throat culture was considered negative for diphtheria bacilli and for beta-hemolytic streptococci, but another one taken the same day was reported positive for *C. diphtheriae*. This strain was found to be virulent. Because of the cervical cellulitis it was hoped that tracheotomy could be avoided, but 6 hours after admission the airway seemed to be nearly obstructed and an emergency tracheotomy was performed. This resulted in a temporary improvement, though the swelling of the neck seemed to be increasing. The pulse was then 120-130 and the respirations 30-35. At that time he was seen by the medical resident, Dr. Newman, who suspected diphtheria, 80,000 units of antitoxin were given intramuscularly and intravenous fluids were administered. The blood pressure had dropped from the previously low level to 66/30. An electrocardiogram taken that night showed marked depression of the S-T segments in leads I and II with elevation in leads III and IV, QRS was splintered in lead IV and T4 was notched. These changes were interpreted as showing widespread myocardial damage which could be due to diphtheria. Intravenous fluids were given slowly and he was also given 50% glucose and 300 mgms of ascorbic acid intravenously. During the next day the patient became steadily worse, the temperature remaining at about 104°, and the pulse dropping to 80. At this time complete heart block was suspected and verified electrocardiographically. During the second night the pulse and blood pressure could not be obtained, the respirations fell to 10 per minute, and the heart rate to 68. The respirations continued to decline, cyanosis increased, and in spite of oxygen and coramine, respirations and heart stopped, death taking place just 36 hours after admission and three and one-half days after the onset of the acute illness.

The *autopsy* showed little on gross examination except dilatation of the heart, some small areas of consolidation in the lungs, and edema of the tissues of the neck. On microscopic examination, patches of diphtheritic membrane were found on the

trachea, there were hemorrhages and edema in the tissues of the neck, and widespread petechial hemorrhages in many organs. The myocardium was not considered abnormal on section except for widespread petechiae, but the adrenals showed very extensive changes, there was a broad band of necrosis in the fascicular layer that extended throughout the cortex. Dr Rich felt that it was very likely that the adrenal lesions were related to the circulatory collapse which had been a prominent feature of the patient's illness.

The anatomical diagnosis was Diphtheria of tonsils, trachea and pharynx. Edema and hemorrhages in the soft tissues of the neck. Hemorrhages in cervical and hilar lymph nodes. Petechial hemorrhages in skin, conjunctivae, pleura, mediastinum, epicardium, myocardium and stomach. Necrosis of the fascicular layer of both adrenals. Foci of lobular pneumonia.

In *summary* this patient had a fulminating and "malignant" form of diphtheria, characterized by a markedly edematous swelling of the tonsils, pharynx and neck, respiratory obstruction requiring tracheotomy, a delayed diagnosis due to the absence of visible membrane, and rapid progression to a fatal termination in spite of antitoxin. There was clinical evidence of myocardial damage which, except for the petechiae, was not apparent on microscopic section, possibly because of its short duration. There were extensive lesions in the adrenals which may have played a role in the fatal outcome.

VII DISCUSSION

As was brought out earlier, diphtheria has shown a marked increase in prevalence in the past few years. This rise lasted for about 3 years in Baltimore and the vicinity, reaching its peak in 1946, during the past year there has been a definite decline again. It is probable that the incidence will decrease for a few years, then rise again as new outbreaks occur.

In view of the widespread nature of the lesions associated with this infection, it is not hard to understand the frequency with which the diagnosis of the primary condition is overlooked. However, if the possibility of diphtheria is kept in mind always in cases of acute respiratory infections, and if the local lesions are observed and cultured carefully, most cases will be recognized at an early stage. The great importance of immediate therapy with antitoxin is brought out in a striking manner by a consideration of the histories of the patients whose cases we have summarized, as well as by those of many more

which have come under observation. The development of a severe form of the disease, with evidence of extensive myocardial damage and often death, seems more closely related to delay in diagnosis and treatment than to any other factor, though the difference in strains and in susceptibility of the host must of course play an important role. If the toxin is not neutralized in the early stages of the infection it is evidently taken up by certain cells, and following this, damage to the cells may progress in spite of the fact that antibodies are present in the blood stream.

The data here presented also illustrate the fact that the clinical manifestations of the severe form of diphtheria may be extremely varied. The majority of these clinical findings may be correlated with and explained on the basis of damage by the toxin to such organs as the heart, the nervous system, or the adrenals. In addition, there may be serious respiratory complications resulting from the local membranous lesions. The cervical edema producing the bull-neck swelling has not been found to be due to bacterial invasion, and at autopsy no evidence of localized infection has been found in the involved tissues. It would seem that this reaction is most likely caused by local spread of the toxin.

It is our hope that this report may stimulate further inquiry into such problems as the adequacy of present immunization procedures. Studies are needed to determine the amount of antitoxin necessary to guarantee an excess in the blood as long as there is circulating toxin. Another interesting problem concerns the mechanism of the toxin's intracellular action. The fact that damage to susceptible cells develops so slowly to the point of clinical recognition—and even more slowly to obvious pathologic changes—suggests that the metabolism of the cells is altered by the diphtheria toxin in a very subtle but progressive manner. These alterations in the metabolic processes at first do not interfere with the cells' normal function to a detectable degree, but when fully developed and sufficiently widespread throughout the involved organ, functional deficiencies become apparent.

The chief purpose of this report is an effort to make the medical profession more alert toward the common occurrence of diphtheria in any community, and in all age groups, whether there is a history of previous immunization or not. Mild cases of the infection are often overlooked

and may get well without specific therapy. On the other hand, in patients who have a more severe type of the disease, any delay in treatment may result in a prolonged and complicated illness or in death. In those with the fulminating or malignant form of diphtheria the early administration of antitoxin is an emergency measure, otherwise such cases progress rapidly to a fatal termination.

VIII SUMMARY

(1) The incidence of diphtheria has shown a decided rise in the past few years, and the severe form has been quite common. (2) The present report deals with the clinical and laboratory features observed in 30 such patients, the object being to illustrate the chief manifestations of the severe or malignant type of the infection. (3) There was clinical evidence of a high incidence of myocardial involvement, this was usually, but not always, verified in the cases in which autopsies were done. (4) Twelve of the 30 patients had a previous history of immunization against diphtheria. Similar experiences have been reported in other outbreaks. (5) The inaccuracy of the virulence test as routinely employed is indicated by the fact that 6 of the 30 strains of diphtheria bacilli isolated were reported as avirulent. (6) The average duration of the acute infection from its onset to the time antitoxin was given was 4.7 days. This delay in therapy was probably the most important cause of serious complications or death. (7) Stress is laid on the urgent necessity of suspecting the possibility of diphtheria in all questionable cases, so that the diagnosis may be made early and specific treatment administered promptly.

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ANTIBODY FORMATION IN ALLOXAN DIABETES

A COMPARISON OF THE PRECIPITIN RESPONSE TO EGG ALBUMEN OF NORMAL RABBITS AND OF RABBITS WITH ALLOXAN DIABETES

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INTRODUCTION

It is generally accepted that patients with diabetes mellitus are more prone to infection than are non-diabetics, but the reason for it is unknown. Possible factors involved have been discussed by Rich (1) under the two following headings: metabolic alterations in the tissue fluids and the state of the mechanisms of resistance. As he points out, previous studies have been made upon human diabetics and totally pancreatectomised animals, in both of which it is difficult to achieve controlled investigation. Alloxan diabetes offered an opportunity to study the problem in animals with presumably normal exocrine pancreatic function and under controlled conditions.

The two main mechanisms of resistance to infection are the formation of antibody and the phagocytic activity of the leucocytes. In this study the first of these mechanisms, as estimated by the precipitin reaction, was investigated in alloxan diabetic rabbits.

EXPERIMENTAL PROCEDURE

Young adult male albino rabbits were made diabetic by the intravenous administration of alloxan (Eastman Kodak) in 4 percent solution in saline. Single doses between 100 mgm per kgm and 200 mgm per kgm were used. No insulin or glucose was administered at any time. Only those rabbits which maintained a blood sugar of about 300 mgm percent (method of Folin and Malmros (2)), or over, with glycosuria were studied. At the time of testing for antibody formation the diabetes had been present for at least four months.

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All the animals were fed the same diet—an unlimited amount of Purina Laboratory Chow and water

Purified crystalline egg albumen, prepared from fresh hens' eggs by the method of Cole (3), was used as the antigen. The rabbits were immunized by four intravenous injections of a 1 percent solution of the crystalline egg albumen at 3 day intervals, 1 c c at the first injection,

TABLE I

EXPT I		Precipitin Test (Antiserum Dilutions)						Weight	Serum Proteins gms %			Blood Sugar	Urine	
Rabbit		1/2	1/4	1/8	1/16	1/32	1/64	Lgm	Total	Alb	Glob	Mgm %	Glucose	Ke tones
Dia- betics	No 7	+	+	+	+	0	0	2 5	5 8	4 2	1 6	348	++++	0
	8	+	+	+	0	0	0	2 9	5 8	4 2	1 6	352	++++	0
	9	+	+	+	0	0	0	3 6	6 1	4 5	1 6	343	++++	0
Nor- mal Con- trols	21	+	+	+	+	0	0	2 7	5 8	3 8	2 0	90	0	0
	22	+	+	+	0	0	0	2 2	5 9	4 1	1 8	90	0	0
	23	+	+	+	0	0	0	2 5	5 3	4 0	1 3	100	0	0
	24	+	+	+	0	0	0	2 6	6 7	4 4	2 3	85	0	0
	25	+	+	+	0	0	0	2 0	5 5	4 3	1 2	85	0	0
	26	+	+	+	0	0	0	2 5	6 4	4 5	1 9	95	0	0
EXPT II														
Dia- betics	A	+	+	+	0	0	0	3 75	5 6	4 4	1 2	308	++	0
	1	+	+	+	+	0	0	3 2	6 3	4 2	2 1	296	+++	0
	6	+	+	0	0	0	0	3 7	5 9	5 1	0 8	328	+++	0
Nor- mal Con- trols	39	+	+	0	0	0	0	4 3	6 3	4 6	1 7	100	0	0
	40	+	+	0	0	0	0	3 5	6 4	4 8	1 6	95	0	0
	41	+	+	+	0	0	0	3 4	5 8	4 2	1 6	110	0	0

2 c c at the second, 4 c c at the third, and 6 c c at the fourth. Ten days after the last injection precipitin tests were done on the serum, using the antiserum dilution method (4). The optimum concentration of antigen for the test was first determined by titrating decreasing concentrations of antigen against undiluted antiserum. The highest dilution of antigen which gave a definite precipitate was chosen for the test. This was found to be $\frac{1}{300}$ of the original 1 percent solution.

Since several of our rabbits had lost weight and were weak, serum

protein determinations were made on all rabbits before immunization and at the time of the precipitin test. Total proteins were determined by the macro-Kjeldahl method (5), and serum albumen and globulin were separated by the method of Kingsley (6).

The diabetic animals were divided into two groups of three. The first group was compared with six normal controls (first part of Table I), the second group with three normal controls (second part of Table I).

RESULTS

Table I summarises the results of this experiment. In the case of the diabetic animals the blood sugar figures quoted are the average of five or six blood sugar estimations carried out during the period of the experiment. Only one serum protein estimation is quoted for each animal, for no significant differences were noted between the preliminary results and those obtained at the time of the precipitin test, the diabetic animals and the normals gave similar values on each occasion.

It will be seen that there is no difference in precipitin titre between the sera of the normal and those of the diabetic animals.

DISCUSSION

Alloxan diabetes in the rabbit is probably not comparable in every respect with human diabetes mellitus, for one thing the rabbit's metabolism is different from that of man, and thus may be reflected in the fact that none of our animals ever became acidotic. At any rate, our rabbits presented continuous hyperglycaemia, glycosuria, polyphagia, polydipsia and polyuria. All of these findings are characteristic of human diabetes mellitus.

Depletion of serum proteins in the rabbit is known to cause a lessened capacity to produce agglutinin when compared with rabbits of similar age (7). In our animals, however, there was no detectable fall in serum proteins, although such a fall was considered quite possible, since several of the rabbits lost weight after they became diabetic.

It was felt that the precipitin test which we used was a sensitive enough index of antibody formation, since the finer differences which might be revealed by more elaborate methods and by hyperimmuniza-

tion would hardly have any practical bearing on the role played by antibody in the increased susceptibility of the diabetic to infections. The use of purified crystalline egg albumen obviated the possibility of any cross reactions which might have been encountered had a substance of more complex antigenic structure been employed.

From our results, therefore, it appears that in the rabbit the alloxan diabetic state by itself does not inhibit the production of antibody to crystalline egg albumen.

SUMMARY

In the absence of any depletion in serum proteins no difference in precipitin titre was found when normal and uncontrolled alloxan diabetic rabbits were immunized with purified crystalline egg albumen.

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COMPARATIVE STUDY OF ANTIHISTAMINE SUBSTANCES¹

I INTRODUCTION AND DALE EXPERIMENTS

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AND

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Interest in recently introduced compounds which have been shown to counteract certain effects of histamine has found expression in a rapidly swelling literature on this subject. It has become increasingly difficult to analyze and compare the various reports on these compounds, which are being offered to the medical profession in steadily increasing number. Obviously many disagreements in the results of animal experiments are due to variations in the technic. Method, dosage, or time of exposure to the drug, these differ from author to author. The small number of animals used by some investigators, the lack of details supplied by others, these impair the value of other reports. The evaluation of results in patients is colored by the inevitable personal factor, because the allergic conditions for which these drugs are administered do not lend themselves to a gauge with which the degree of improvement can be reliably measured.

We attempted to compare a number of the antihistamine substances with uniform methods in a considerable number of animal experiments. In order to obtain an unbiased picture of their clinical value, the use of each drug was assigned to several members of our clinic group.

The solutions used for the animal experiments were made in our laboratory from the powdered drugs which we obtained through the courtesy of the various pharmaceutical houses. The French compound 3277RP was supplied through the kindness of Prof. Pasteur Vallery-Radot (Paris). For clinical use the drugs were available in capsules or tablets, furnished in generous amounts by the cooperating firms.

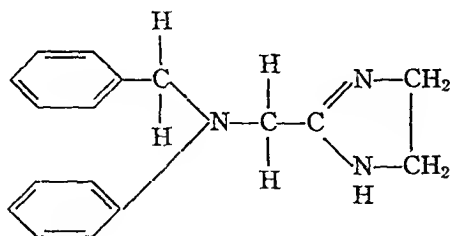
¹ This investigation has been aided by grants from G. D. Searle and Company, Eli Lilly and Company and the Houston Endowment, Incorporated.

² From the Protein Clinic of the Department of Medicine, The Johns Hopkins Hospital.

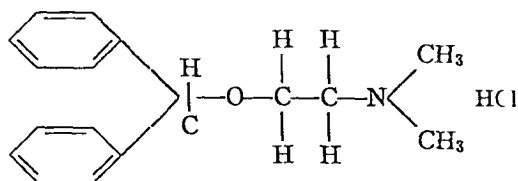
No data relative to the stability of these substances and their solutions have been published so far. Recently, however, one manufacturing company gave the information that the antihistamine drug they offer for clinical trial discolours with exposure to sunlight and interacts with rubber. In preliminary tests with the drugs, we found that the potency of higher dilutions of several drugs decreased considerably, even when these dilutions were kept in the refrigerator. In one instance, we found that 3277RP had oxidized en route from France. Detailed studies relative to the stability of the various antihistamine substances are urgently needed.

Various methods of testing these drugs in animals are available, and have been used. We believed that observation of their action on the intestinal strip of guinea-pigs and of their protective effects in guinea-pigs *in vivo* would give us a sufficiently representative picture of their comparative potency.

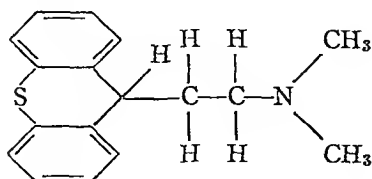
The compounds which we have used in the animal experiments are Antistine (Ciba), Benadryl (Parke, Davis & Co.), 1721 (Searle & Co.), 3277 (Rhone Poulenc), Histadyl (Eli Lilly & Co.), Chlorothen (Lederle), Bromothen (Lederle), Pyribenzamine (Ciba), Neo-Antergan



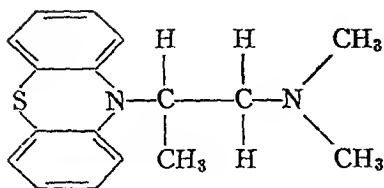
ANTISTINE



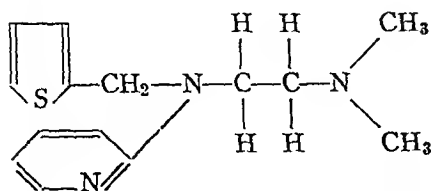
HCl

BENADRYL
(Diphenhydramine
HCl)

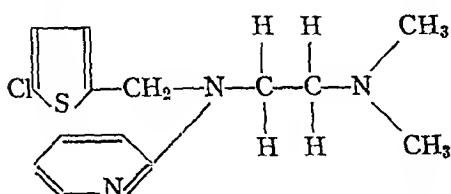
1721 (SEARLE)



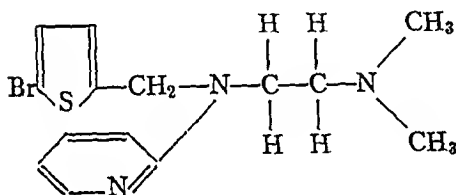
3277 R P



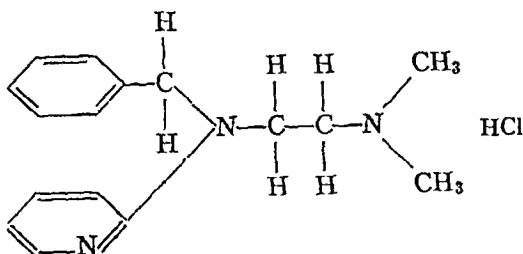
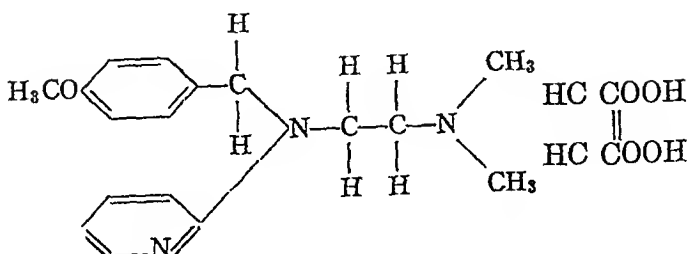
HISTADYL



CHLOROTHEN



BROMOTHEN

PYRIBENZAMINE
(Tripelenamine
HCl)NEO-ANTERGAN
(Pyranisamine
Maleate)

(Merck & Co) (See structure formulas) Antistine is a Swiss preparation, 3277 and Neo-Antergan are French compounds, all the other drugs have been developed in the United States In the clinical trials

we used all these drugs except 3277, and, in addition, we used Hydrylin (Searle), a mixture of aminophyllin and diphenhydramine base, and 1913 (Searle), a mixture of aminophyllin and 1721 (Searle). In preliminary screening tests, we had also used two other Searle preparations, 1694, a xanthin salt of Benadryl, and 1695, a xanthin salt of the French compound 3015.

DALE EXPERIMENTS

(a) *Method* The bath containing the suspended intestinal strip, along with a 3-liter flask containing Tyrode solution, was kept immersed in a 5-gallon battery jar. The water in this jar was agitated by a stirring motor, and its temperature was kept constant by an electric thermostat. Oxygen 95% with CO₂ 5% bubbled through the bath by means of a gas disperser sealed in the bottom of the bath cylinder. Drainage was conducted through another opening underneath.³ Most workers employ a bath temperature of 37.5° or 38° Centigrade. Our observations seemed to indicate that a temperature of 36° Centigrade gave better working conditions. Frommel and co-workers (1) found a temperature of even 34° Centigrade optimal.

The Tyrode solution was prepared according to Burn (2), with the exception that solution B contained 0.25% NaHCO₃. We had had difficulties in maintaining the pH when we used Burn's original formula, and found this modification used by Halpern (3) more satisfactory.

In all our experiments, contraction of the strip was achieved by adding 10 gamma of histamine⁴ to 100 cc of the bath (0.1 gamma per cc). In guinea-pigs of adequate sensitivity a maximal contraction is obtainable with this dose. When submaximal contractions resulted, the strip was discarded. We found, as other authors had found, that not only do guts of different guinea-pigs vary considerably in sensitivity but also segments of the same ileum vary.

Twenty years ago Kendall and collaborators published extensive

³ We are indebted to Dr. A. H. Corwin (Chemistry Department, Johns Hopkins University) and to Dr. J. E. Cushing (Biology Department, Johns Hopkins University) for invaluable help and advice in setting up the apparatus.

⁴ All doses of histamine are expressed as base. Histamine acid phosphate (Lilly or Burroughs Wellcome) was used.

studies on intestinal strips of guinea-pigs Kendall and Varney (4) stated that their results "never became mathematical in their precision" They predicted that perfection would never be achieved until some of the idiosyncrasies of smooth muscle are better understood This statement is equally true today, and should be kept in mind On the basis of more than 600 Dale experiments, we learned to be extremely cautious in our evaluation of the tests Recently, Chen and co-workers (5) have rightly stressed the erratic behavior of the gut in many instances

In 1939 Anne-Marie Staub (6) pointed out that the effective concentration of the antihistamine 929F varied from one gut to another She further observed that after suppression of the histamine reaction with 929F, washing out of the gut did not completely restore its activity Even when the muscle contraction maintained the same intensity, such contraction could be more easily suppressed than before According to Staub, subsequent tests are therefore not of equal value with the first test

For several weeks, we worked with Staub's technic, i e , *inhibition* of histamine contraction by preceding addition of the antihistamine to the bath, and experienced the following difficulties

- 1 At times the histamine contraction achieved after repeated washing of the strip following the test would be higher than the contraction preceding the test From which contraction should the percentage of inhibition be calculated?

- 2 With the more potent drugs, particularly Neo-Antergan, Chlorothen, and the slow-acting 3277, the gut failed indefinitely to regain its previous sensitivity to histamine Therefore, we could not ascertain whether the gut had the same sensitivity during this test as that shown in the preceding histamine contraction

- 3 In many instances we observed a two-phasic effect When histamine was given after the antihistamine, there resulted a contraction to a certain height followed immediately by relaxation to a certain point In such cases how should the degree of inhibition be calculated?

Because of these uncertainties we adopted Halpern's (3) technic, which estimates the degree of *relaxation* caused by the addition of the antihistaminic drug to the bath, after the histamine contraction has taken place Using this method, we arrived at the same conclusion as

Schild (7) It is preferable to use a new piece of gut for each test. Washing the gut until the original height of histamine contraction is restored may involve as much time as suspending a new strip. If Staub's (6) observation is valid, even the same response to histamine as before does not guarantee the same sensitivity to the antihistaminic substance.

Therefore, the procedure finally chosen was as follows: after suspension of the strip, 0.1 gamma/cc histamine base was added to the Tyrode solution. If a maximal contraction was obtained, the strip

TABLE 1

Action of various antihistamine drugs on the histamine contraction (0.1 gamma/cc) of the intestinal strips of guinea-pigs

Exposure to histamine and to drug each one minute

DRUG	0.01 GAMMA/CC	0.02 GAMMA/CC	0.05 GAMMA/CC	0.1 GAMMA/CC	0.2 GAMMA/CC
Antistine	12	25	52	88	97
Benadryl	38	71	83	96	
1721	32	56	81	90	
3277RP	30	55	73	84	
Histadyl	93	97			
Bromothén	85	92			
Chlorothén	98				
Pyribenzamine	81	95			
Neo Antergan	73	97			

NOTE: Numbers indicate percentage of relaxation.

was washed and a second histamine contraction elicited. One minute later the antihistamine compound was added to the bath. (Histamine, as well as its antagonist, was always contained in the same volume of distilled water, one cc.) After conclusion of the test, the strip was discarded and a new piece of gut suspended.

(b) *Results* Table 1 shows the percentage of relaxation (from the histamine contraction) accomplished by varying amounts of each drug when the drum was stopped one minute after the drug had been added. No test was accepted for calculation of the percentage when the slightest doubt existed as to the normal sensitivity of the gut. Each number in the first column (0.01 gamma/cc) represents the average of eight to

twelve determinations, each number in the other columns represents the average of four to seven determinations

Antistine proved to be of lowest potency Benadryl, 1721, and 3277 had about equal moderate effectiveness, Benadryl's being slightly higher than the other two Histadyl, Bromothen, Chlorothen, Pyribenzamine, and Neo-Antergan can be grouped together, for 0.01

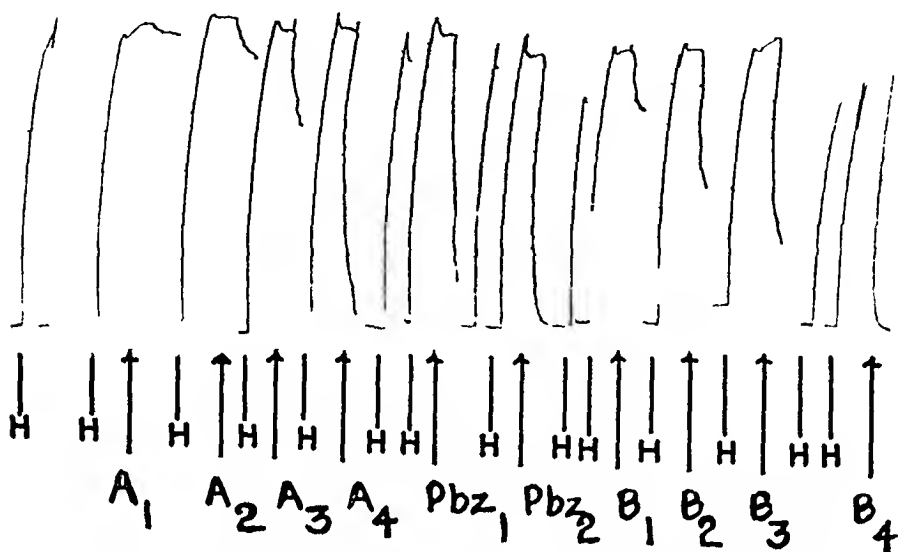


FIG 1 Dale test, intestinal strip of guinea pig Exposure to histamine one minute, to antihistamine substance one minute

H = 0.1 gamma/cc histamine base

A1 = 0.01 gamma/cc Antistine

A2 = 0.02 gamma/cc Antistine

A3 = 0.05 gamma/cc Antistine

A4 = 0.1 gamma/cc Antistine

Pbz 1 = 0.01 gamma/cc Pyribenzamine

Pbz 2 = 0.02 gamma/cc Pyribenzamine

B1 = 0.01 gamma/cc Benadryl

B2 = 0.02 gamma/cc Benadryl

B3 = 0.05 gamma/cc Benadryl

B4 = 0.1 gamma/cc Benadryl

to 0.02 gamma/cc of these compounds caused almost complete relaxation of the contracted muscle within one minute (Table 1, fig 1 and 2)

Table 2 shows that the differences in the most potent group are insignificant When we determined the time required to reach base line after addition of the antagonist, we found that the activity of the drugs in this group was very similar

On closer analysis, the apparent superiority of Benadryl over 3277

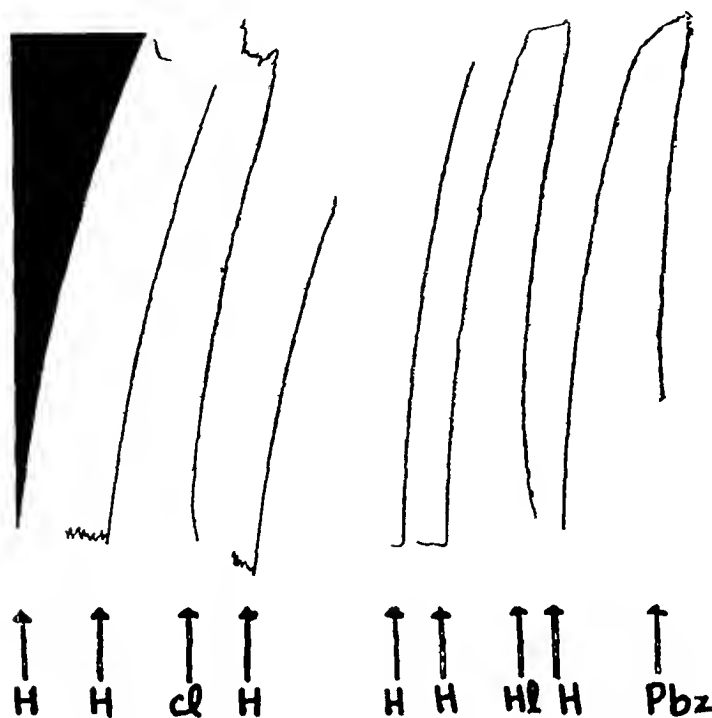


FIG 2 Dale test, intestinal strip of guinea pig Exposure to histamine one minute, to antihistamine substance one minute

H = 0.1 gamma/cc histamine base

Hl = 0.01 gamma/cc Histadyl

Cl = 0.01 gamma/cc Chlorothen

Pbz = 0.01 gamma/cc Pyribenzamine

TABLE 2

Time required for 100% relaxation of the intestinal strip contracted with 0.1 gamma/cc histamine for 5 minutes

DRUG (0.01 GAMMA/CC)	TIME
	<i>seconds</i>
Histadyl	85
Chlorothen	105
Pyribenzamine	100
Neo Antergan	90

disappeared. The French preparation was found to have much slower action than the other compounds. After adding 3277 to the bath, from 30 to 40 seconds was required for relaxation to begin in contrast

with 5 to 8 seconds required by the other substances. Correspondingly, if the strip was exposed for 5 minutes to 3277, 0 01 gamma/cc achieved almost complete relaxation, whereas, the relaxation after a 5 minute exposure to Benadryl (0 01 gamma/cc) averaged 65%. In a five-minute exposure, the same dose of Antistine did not produce more than 42% relaxation (Fig 3a)

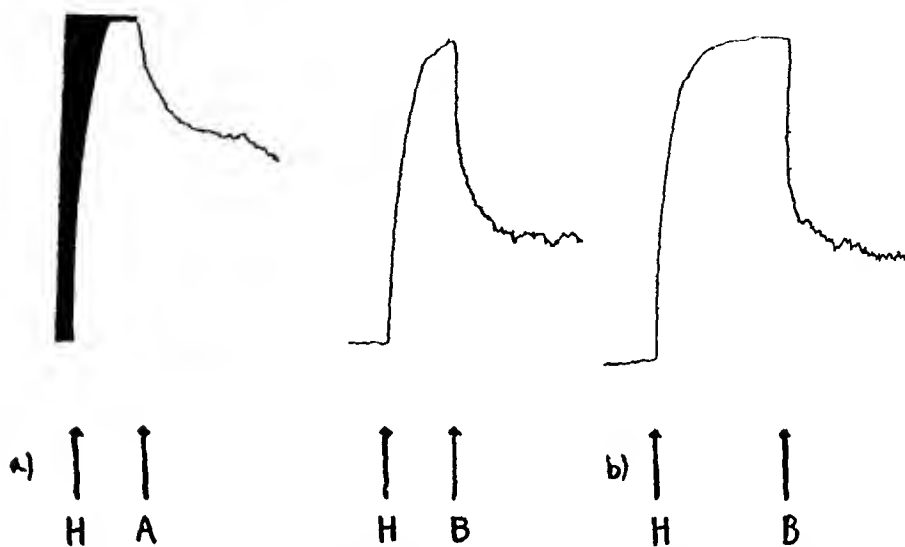


FIG 3a Dale test, intestinal strip of guinea pig Exposure to histamine one minute, to antihistamine substance five minutes

FIG 3b Same Exposure to histamine five minutes, to antihistamine substance five minutes

H = 0 1 gamma/cc histamine base

A = 0 01 gamma/cc Antistine

B = 0 01 gamma/cc Benadryl

When we allowed histamine to act on the gut for five minutes instead of one, the relaxation due to the following antagonist was not appreciably changed in any group. This is illustrated in Fig 3b and Fig 4.

(c) *Discussion* Comparison of our results with results reported in the literature is difficult because of the lack of uniform procedure. Some authors used histamine diphosphate, others used histamine dihydrochloride. Certain writers express their histamine dose in terms of the salt, others express dosage in terms of histamine base, a few neglect to give this necessary information. It is confusing to find

figures of "histamine" concentration in the bath, tabulated without the author's taking account of the fact that the figures of one investigator represent histamine dihydrochloride but those of the second investi-

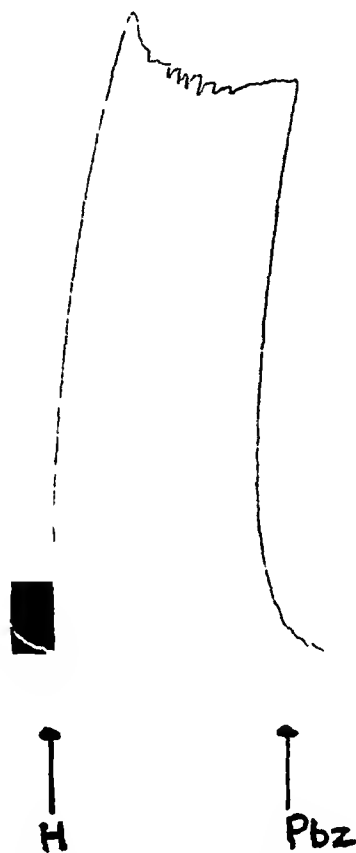


FIG 4 Dale test, intestinal strip of guinea pig Exposure to histamine five minutes, to antihistamine substance two minutes

H = 0.1 gamma histamine base

Pbz = 0.01 gamma Pyribenzamine

gator represent histamine diphosphate (dihydrochloride contains 60% histamine base, diphosphate contains 36% histamine base)

Table 3 is a compilation of quantitative data presented in sufficient detail to enable one to calculate the action of a single drug against histamine. The figures of the authors, given in terms of histamine salt, have been changed to histamine base values. Benadryl counter-

acted approximately an equal amount of histamine Neo-Antergan and Pyrribenzamine were effective in counteracting 6 to 10 times as much histamine Histadyl was effective against 18 to 72 times as much histamine, however, this drug was allowed to act two minutes on the gut

Data comparing the activity of several compounds are few and conflicting Taking the average from 12 to 16 experiments, Winter (11) determined the inhibition for each of five drugs He found this percentage to be Neo-Antergan 81, Pyrribenzamine 68, 3277, 45, Benadryl 42, Hetramine 11 Graham (13) found the quantity of various compounds required *to reduce by one-half* the spasm produced by

TABLE 3

Results in the literature of effective counteraction of histamine contraction in the Dale bath

AUTHOR	METHOD USED	HISTAMINE gamma/cc	ANTIHISTAMINE DRUG		TIME OF CONTACT
			Kind	gamma/cc	
Loew et al (8)	Inhibition	0 02 appr	Benadryl	0 02-0 04	one min
R Mayer (9)	Inhibition	0 036-0 36	Pyrribenzamine	0 02-0 04	one min
Bovet et al (10)	Relaxation	0 06	Neo-Antergan	0 01	?
Winter (11)	Inhibition	0 12	Neo Antergan	0 02	one min
Lee et al (12)	Relaxation	0 18	Histadyl	0 0025-0 01	two min

1 gamma/75 cc histamine to be Neo-Antergan 0 07, Benadryl 0 7, Antistine 5 3 In his experiments the drug was allowed to act on the gut for two minutes Lee and collaborators (12), who also exposed the gut for two minutes to the antagonist, found Histadyl (01013) on the average 4 9 times as active as Benadryl, but 0 8 as active as Pyrribenzamine

Most of our results are similar to those determined by Winter (11) When 0 01 gamma/cc was used, Neo-Antergan was twice as effective as Benadryl We found 3277, if the effect in one minute is measured, in the same group as Benadryl But in our tests, Neo-Antergan was somewhat less potent than Pyrribenzamine In marked contrast to Lee and collaborators (12), Histadyl in small doses and on short contact was more effective than Pyrribenzamine

(d) *Conclusions* In view of the present uncertainties and the m-

evitable inaccuracies in this kind of determination, we feel that not much weight should be given to small differences. Conservatively, we can state these results. Within one minute, Histadyl, Bromothen, Chlorothen, Pyribenzamine, and Neo-Antergan completely counteracted five times as much histamine. Within one and a half to one and three-quarter minutes, these drugs counteracted ten times as much histamine. Within one minute, Benadryl completely antagonized an equal amount of histamine but Antistine was fully effective against only one-half the amount of histamine. The French preparation, 3277, differed from the other compounds in its slow action. *In five minutes* this drug was able completely to antagonize ten times as much histamine.

In the literature there are very few reliable data concerning the degree to which antihistaminic drugs counteract the contraction produced by the homologous antigen in a sensitized guinea-pig strip. Available data indicate that larger doses are required to be effective against antigen contractions than are required to be effective against histamine contractions (Staub (6), LaBarre et Reuse (14), R. Meier (15)). Our preliminary tests, to be extended and published later, confirm these observations. Rose and collaborators (16) came to the opposite conclusion. They used strips from guinea-pigs passively sensitized, and no statement was made concerning the number of their tests.

SUMMARY

Our study of the action of 9 antihistamine substances in Dale tests revealed three groups of potency. Antistine was weakest, Benadryl, 1721 (Searle), 3277RP were moderate, Histadyl, Chlorothen, Bromothen, Pyribenzamine and Neo-Antergan were highly potent.

These values are based on one-minute contact of the smooth muscle with the drug. In prolonged contact 3277 equaled the effectiveness of the drugs in the most potent group.

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COMPARATIVE STUDY OF ANTIHISTAMINE SUBSTANCES¹

II ACTIVITY IN VIVO AGAINST HISTAMINE INTOXICATION AND ANAPHYLACTIC SHOCK OF GUINEA PIGS

S WALTER LANDAU, HENRY J L MARRIOTT, AND LESLIE N. GAY²

A HISTAMINE INTOXICATION

The antagonizing effect of the antihistamine substances against the bronchoconstrictor action of histamine in the intact guinea pig has been examined through various procedures Staub (1), a pioneer in the field, injected a fixed dose of the antihistamine compound prior to the intravenous administration of histamine, and observed the number of lethal doses of histamine that the animals would tolerate Subsequently, a number of investigators employed Staub's technique Halpern (2, 3), using this method, compared a large series of compounds, and with his latest preparation (3277) arrived at the figure of 1500 lethal doses of histamine against which 20 mg of 3277 would protect

The same author inaugurated the testing of the drugs against the effect of aerosoled histamine In a few experiments he gave the antihistamine compound per os, in others he mixed it with histamine in the aerosol, in most experiments he administered the antihistamine subcutaneously at various intervals before the exposure to histamine aerosol This latter technique has been adopted by many investigators Halpern (3) observed the time until the appearance of severe asthma, and considered those animals who had no symptoms after 10 minutes of inhalation as protected Mayer (4) and Winter (5) also employed this technique to test reduction of or freedom from symptoms A significant reduction in the mortality rate was taken as the indicator by other authors (Loew and co-workers (12), Litchfield and co-workers (6), Meier and Bucher (7), Lee and co-workers (8))

The third method employed consists of determining the protection

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² From the Protein Clinic of the Department of Medicine, The Johns Hopkins Hospital

created by prior administration of the antihistamine compound to resist one lethal dose of histamine injected into the bloodstream Winter (5), as well as Rose and collaborators (9), has studied the action of histamine antagonists in this manner. Independently, we had chosen this technique before we started our comparative study. We believe that death from the minimal lethal dose of histamine as the indicator eliminates any subjective factor from evaluation of the protective effect, and that parenteral administration of drugs best guarantees the introduction into the animal of the dosage selected for the experiment, an all-important factor in quantitative studies.

(a) *Method* Our aim was to determine the smallest dose of the various compounds that would prevent death from one lethal dose of histamine in 100% of the animals. This minimal lethal dose was again established as 0.5 mg/kg histamine base when injected intracardially (Landau and Gay (10)). The procedure was as follows. In a first set of experiments 0.5 mg/kg histamine was injected into the heart, thirty minutes after the subcutaneous injection of an antihistamine compound. In further series of experiments, the dose of this compound was increased or decreased depending on the percentage of surviving animals. With some drugs we could base our initial dose on reports in the literature, with others we had to find our way slowly to the protective dose.

Interested in the duration of the protective effect, we injected guinea pigs intracardially with one lethal dose of histamine two hours after the subcutaneous administration of that dose of the compound that had given 100% protection after half an hour. Those drugs that gave significant protection after two hours were chosen for determination of their effect after five hours.

All the animals were observed for severity of symptoms. In each case of death, an autopsy was performed. In a number of animals, the dissection showed considerable hemorrhage from the heart. We believed that in these cases the violent convulsions effected by histamine had caused bleeding from the site of the heart puncture,³ consequently, these deaths were due to a combination of causes: intracardiac injection plus reaction to histamine. Therefore, we excluded

³ Intracardial injection of salt solution produced no trouble in ten animals

from the series those animals in which we found such a major hemorrhage. We had followed the same practice in our work on arginine and histidine. A survey showed that if these animals had been included, the total results would have lowered some of our figures of survival by 5% to 15%, but would not have influenced the order of potency of the different drugs.

(b) *Results* Table 1⁴ shows the degree of protection from varying doses of nine antihistamine compounds, injected one-half hour prior to the lethal dose of histamine. The minimal 100% protective doses are in mg/kg: Antistine 1.5, Benadryl 0.6, drug 1721 (Searle) 0.4, drug 3277RP 0.2, Bromothen 0.05, Chlorothen 0.05, Histadyl 0.05, Pyribenzamine 0.04, Neo-Antergan 0.02. The percentage of animals that show less than three-plus symptoms runs almost parallel to the increasing percentage of survival. When a multiple of the minimal dose that protected 100% of the animals was given, as in the case of Chlorothen and Bromothen, the animals remained practically free from symptoms.

Table 2 indicates the duration of the protective effect. After two hours no appreciable protection remained in the series with Antistine, Benadryl, and Histadyl, from 60% to 100% protection remained in the series with 1721, Bromothen, Chlorothen, Pyribenzamine, 3277 and Neo-Antergan. After five hours this remaining activity had completely subsided in the Chlorothen series and had become insignificant in the Pyribenzamine and Neo-Antergan series. However, 73% of the animals injected with 3277 survived one lethal dose of histamine injected five hours later.

(c) *Discussion* The order of potency against histamine in these in vivo experiments parallels and confirms the results of our Dale tests in the following characteristics. Antistine requires the largest dose for protection, Histadyl, Chlorothen, Bromothen, Pyribenzamine and Neo-Antergan are effective in very small doses, Benadryl, 1721 and 3277, when we consider the protection within 30 minutes, occupy a middle place. In this middle group, Benadryl is definitely inferior to 1721 and 3277. In contrast to the Dale results Neo-Antergan emerges as the most potent drug, its minimal 100% protective dose being half

⁴ The figures are tabulated as in Winter's (5) study to facilitate comparison.

TABLE 1

Protection of guinea pigs with antihistamine compounds, given subcutaneously, against one lethal dose of histamine, given intracardially 30 minutes later

ANTIHISTAMINE COMPOUND (SUBCUTANEOUSLY)		SEVERITY OF SYMPTOMS* (NUMBER OF ANIMALS)					SURVIVAL %	LESS THAN 3+ SYMPTOMS %
Kind	Dose	0	+	++	+++	Death		
None (controls)	mg/kg	0	0	0	1	9	10	0
Antistine (62 animals)	0 01	0	0	0	0	10	0	0
	0 04	0	0	0	0	8	0	0
	0 5	1	1	1	2	5	50	30
	1 0	0	2	3	2	3	70	50
	1 5	0	1	4	8	0	100	39
	2 0	2	5	1	2	0	100	80
Benadryl (43 animals)	0 2	0	0	2	7	3	75	17
	0 4	0	1	3	4	2	80	50
	0 5	0	2	3	4	1	90	50
	0 6	0	3	4	4	0	100	64
1721 (Searle) (45 animals)	0 1	1	0	0	0	8	12	12
	0 2	0	2	0	1	9	25	18
	0 3	0	3	1	6	2	83	33
	0 4	1	2	2	7	0	100	42
3277 RP (22 animals)	0 1	0	1	2	5	2	80	30
	0 2	6	2	2	2	0	100	85
Bromothén (73 animals)	0 01	1	0	0	3	6	40	10
	0 02	0	2	5	5	2	86	50
	0 04	0	0	6	7	3	81	37
	0 05	0	4	0	7	0	100	36
	0 06	0	3	5	4	0	100	67
	0 2	9	1	0	0	0	100	100
Chlorothén (93 animals)	0 005	0	0	0	1	6	14	0
	0 01	0	0	3	12	1	93	18
	0 02	1	3	5	9	2	90	45
	0 04	1	5	4	6	2	89	62
	0 05	0	3	2	5	0	100	50
	0 06	0	3	3	6	0	100	50
	0 2	9	1	0	0	0	100	100
Histadyl (41 animals)	0 04	0	1	1	1	7	30	20
	0 05	0	1	5	4	0	100	60
	0 06	2	2	3	3	0	100	70
	0 1	4	4	1	2	0	100	90

TABLE 1—*Continued*

ANTIHISTAMINE COMPOUND (SUBCUTANEOUSLY)		SEVERITY OF SYMPTOMS* (NUMBER OF ANIMALS)					SURVIVAL %	LESS THAN 3+ SYMPTOMS %
Kind	Dose	0	+	++	+++	Death		
	mg/kg							
Pyrribenzamine (34 animals)	0.02	0	0	0	5	7	42	0
	0.03	0	0	2	8	1	91	18
	0.04	0	1	4	6	0	100	45
Neo Antergan (32 animals)	0.005	0	0	0	5	5	50	0
	0.01	0	2	2	6	1	91	36
	0.02	3	3	4	2	0	100	85

* 0 = no symptoms, + = accelerated respiration, itching, ++ = cough, asthma, +++ = convulsions, weakness

TABLE 2

Duration of protection with 100% effective dose of antihistamine compounds (subcutaneously) against one lethal dose of histamine (intracardially)

ANTIHISTAMINE COMPOUND	AFTER 2 HOURS			AFTER 5 HOURS	
	Number of Animals	Survival %	Less than 3+ Symptoms %	Number of Animals	Survival %
Antistine	7	14	0		
Benadryl	6	16	0		
1721 (Searle)	10	60	0		
3277RP	10	100	90	11	73
Bromothén	19	84	36		
Chlorothén	21	81	24	5	0
Histadyl	7	14	14		
Pyrribenzamine	19	84	36	7	30
Neo-Antergan	19	100	47	4	25

as much as that of Pyrribenzamine and less than half as much as that of the other drugs in the same group

Winter (5), using the same technique with the exception that he gave histamine intravenously, tested six compounds, four of which are included in our study. He, likewise, found Neo-Antergan to be the most potent drug, followed by Pyrribenzamine. His 100% protective dose of Neo-Antergan was 0.01 mg/kg. With this dose we had 91% survival, an insignificant difference, particularly so in view of the fact

that the percentage of animals with less than three-plus symptoms was almost the same in his and our series. Our figures for Pynbenzamine are slightly less favorable than Winter's. In this author's studies, 3277RP was definitely more effective than Benadryl, the dose for 100% survival, however, was not determined for these two drugs.

Figures for Histadyl have been published by Feinberg and Bernstein (11). They achieved 100% protection by intraperitoneal injection of 0.1 mg/kg, but only 50% protection with 0.05 mg/kg. Rose and co-workers (9) also injected the antihistamine substance intraperitoneally and administered the lethal dose of histamine as early as fifteen minutes after the protective agent. It may be due to these differences in procedure that in their studies as much as 0.1 mg/kg Neo-Antergan gave only 78% protection. With Benadryl, Rose and co-workers (9) still had, surprisingly, a mortality of 29% with a dose of 3 mg/kg.

When we compare our results with those of authors who used aerosoled histamine, we find that Sherrod and co-workers (12) in their studies determined the potency of Neo-Antergan to be four times and twenty times, respectively, higher than that of Pynbenzamine and of Benadryl. These results refer to intraperitoneal injection of the antihistamine substance and to its ability significantly to reduce the incidence of mortality. Their conclusion might appear as too unfavorable towards Pynbenzamine, for with 0.3 mg/kg of this drug their reduction of mortality was 65% compared with 20% reduction with 1.5 mg/kg Benadryl and 30% reduction with 0.075 mg/kg Neo-Antergan.

The comparatively weak activity of 3277 in our studies, as well as in Winter's, is surprising in view of the fact that Halpern found 3277 far superior to Neo-Antergan, when these drugs were used against multiple lethal doses of histamine. 3277 protected, as mentioned, against 1500 lethal doses, whereas Neo-Antergan protected against only 80 lethal doses. Winter tested the effect of both drugs against massive doses of histamine, but found no impressive difference, however, the number of animals used was small. Halpern's figures have not been confirmed by other workers in France. Lerman and Goldfeder (13) noted that the protection against aerosoled histamine seemed to be more complete with Neo-Antergan than with 3277.

As to the duration of protection, our results confirm Halpern's statement, made on the basis of aerosol experiments, that 3277 dis-

tinguishes itself by its prolonged effect. In our studies, this drug was the only one that had lost very little of its action within five hours. Litchfield and co-workers (6) found the duration of protection against aerosoled histamine shorter for Thenyl (Histadyl) than for Pyrribenzamine. A corresponding difference between these two drugs was obvious in our series. These authors also found that with the same method, Chlorothen and Bromothen each gave protection for twice as long as did Pyrribenzamine. We found that with these three drugs, the rate of survival was equal after two hours and no significant effect was present with either of the compounds after five hours.

B ANAPHYLAXIS

(a) *Method* In a large majority of our anaphylaxis experiments, sheep serum was used as antigen. A single sensitizing dose (0.5 cc) was given intraperitoneally, the challenging dose (0.25 cc) was given intracardially in most series, in a few series with Neo-Antergan the challenging dose was injected into the jugular vein. (See table 3) With this technique 86% of the controls died from anaphylactic shock. In some series we employed egg white as antigen, giving 2 cc of a 10% solution as sensitizing dose and 0.5 cc of a 2% solution as challenging dose, all these controls (12) died in anaphylactic shock. In table 3 all figures without an asterisk denote sheep serum as the antigen. Twenty-one days after sensitization, the challenging dose was injected into all animals. The antihistamine drug was administered, subcutaneously, thirty minutes prior to the reinjection of the antigen. In all cases of death, autopsies were done and animals with major hemorrhage from the heart were discarded, as in the histamine experiments.

(b) *Results* Antistine again proved to be the weakest agent in animal experiments. With 10 mg/kg, only 64% of the guinea pigs were protected against anaphylactic shock. In striking contrast to the histamine studies, the other drugs exhibited almost equal effectiveness, inasmuch as a dose of 3 or 4 mg prevented anaphylactic death in from 90% to 100% of the animals. Certain differences among the drugs appear in the figures, but on a minor scale. (See discussion.)

A number of animals that had survived anaphylactic shock, because of protection by an antihistamine substance, received a second shocking dose 36 hours later. The results were as follows. Of 11 animals that

Antistine or 3277 had protected against one shocking dose, 3 died in anaphylactic shock and 6 had typical shock symptoms, i.e., 82% of the animals could be successfully shocked. Of 8 animals that Chlorothen

TABLE 3

Protection of guinea pigs with antihistamine compounds against active anaphylaxis

ANTIHISTAMINE COMPOUND		NUMBER OF ANIMALS	NUMBER OF DEATHS	SURVIVAL %
Kind	Dose			
	mg/kg			
None (Controls)		12x	12	0
None (Controls)		43	37	14
Antistine	2	4x	4	0
	4	5x	5	0
	10	14	5	64
Benadryl	3	10	0	100
1721 (Searle)	2	10x	3	70
	3	11	4+	64
3277RP	2	9x	3	66
	3	10	2+	80
	4	13	1+	92
Bromothen	3	9	0	100
Chlorothen	3	10	0	100
Histadyl	2	6x	2	66
	3	10	1	90
Pyribenzamine	2	8x	8	0
	3	20	7+	65
	4	9	0	100
Neo-Antigen	2	6	5	16
	3	19 ¹⁾	9	52
	4	10 ²⁾	2	80
	5	9 ²⁾	1+	88

x = egg white, as antigen

+ = delayed death

¹⁾ = in 12 animals, antigen injected intravenously

²⁾ = antigen injected intravenously

or Histadyl had protected against two shocking doses, 4 died in anaphylactic shock and 4 had typical shock symptoms. In summation, thirty-six hours after survival from one or two shocking doses, because of protection by one of the antihistamine compounds, 17 of the 19 animals (89%) responded to another shocking dose, 7 animals (36%) with fatal shock.

(c) *Discussion* Most investigators have examined the compounds at their disposal only for their activity against histamine, therefore, very few figures derived through similar technique, and thus suitable for comparison, are available in regard to their anti-anaphylactic effectiveness. Again our results are in sharp disagreement with Halpern and co-workers (14). These authors, using 1 mg/kg Neo-Antergan (4 animals) and 0.25 mg 3277 (6 animals) reported 100% survival from active anaphylactic shock induced by sheep serum. With 0.1 mg 3277, they obtained 80% survival in ten animals. Arbesman and co-workers (15), as well as Rose and coworkers (9), tested some compounds against *passive* anaphylaxis and achieved protection with somewhat smaller doses of Pyribenzamine and Neo-Antergan than we found necessary in our studies. Mayer and co-workers (16), who used Pyribenzamine spray preceding the shocking dose, likewise found the protection greater and more consistent in animals passively sensitized than in those actively sensitized. How difficult it is to obtain identical results with identical methods is seen by comparing the figures of Rose and co-workers (9) who had 70% survival following 2 mg/kg Pyribenzamine and 100% survival following 3 mg/kg of this drug, with those of Arbesman and co-workers (15), who (in a small series) had 100% survival following 2 mg/kg Pyribenzamine. Therefore, too much importance should not be attached to differences which might disappear by increasing the number of animals in a given series.

Lehman and Young (17), working with diethylaminoethyl-dihydroanthracen-carboxylate, observed that sensitized guinea pigs which this substance had protected against 50 times as much antigen as had killed 9 of 10 controls did not show any symptoms when they received a subsequent injection of the same dose of antigen two days later. They concluded that the animals had been desensitized by the first shocking dose, therefore, they presumed that the protective effect of their compound was due to its antihistaminic action. Mayer (16) noticed that after protection with Pyribenzamine against anaphylaxis, the animals were still desensitized, and persisted in a state of antianaphylaxis eight days after the first antigen dose. A "silent antibody reaction" seems to have taken place. Such an explanation would be in accordance with observations on the sensitized intestinal strip formerly made by Ackerman and Wasmuth (18) and by Landau and Gay (10), using arginine as

protective agent, and by Rosenthal and Brown (19), using 929F Ackerman and Wasmuth (18) suggested that the antigen-antibody reaction takes its course unimpeded by arginine, but that the amount of histamine liberated by the antigen-antibody reaction is counteracted. Our own antianaphylaxis studies failed to confirm these *in vivo* observations, for 36 hours after survival from anaphylactic shock because of protection with various compounds, 17 of the 19 guinea pigs could be shocked. Staub, Halpern, and Vallery-Radot and co-workers (20) reported the same observations. The last named authors repeated the shocking dose after 80 hours and obtained reactions, hence, they concluded that the injection of 3277 had inhibited not only the manifestations of shock but also the reaction of the antigen with the antibody. Staub had obtained fatal shock within one, two, or seven days after the animals had survived the first shocking dose, because of protection with 929F. In view of the fact that Lehman and Young (17) obtained their widely different results by using very large doses of antigen, a quantitative factor might be involved.

CONCLUSIONS

Our studies show that there is a wide difference in potency among these nine so-called antihistamine compounds when tested against one lethal dose of histamine injected into the bloodstream. The scale of effectiveness approximates the activity against the histamine-induced contraction of the intestinal strip of guinea pigs. In striking contrast, all the substances except Antistine proved to be almost equally effective against anaphylaxis. The dose required for protection against anaphylactic death was considerably higher than that required for protection against one lethal dose of histamine, naturally, this difference became the greater, the smaller the protective dose against histamine. The need for higher protective dosage against anaphylaxis than against histamine has been stressed before by two of the present writers (10) and it has been reiterated by Lehman and Young (17).

We believe that our comparative quantitative studies supply the evidence which Loew (21) felt to be lacking to prove this point. Loew (21) stated that this difference in the required dosage would not constitute evidence that factors other than histamine were involved to an important degree in anaphylaxis. On the basis of our observations

with arginine and histidine, we ourselves suggested "that histamine liberated by the antigen-antibody reaction is in more intimate association with the tissue," consequently, more effective than histamine added from the outside

Why is the potency against histamine so widely different, but the dosage for antianaphylactic effect so very similar? Could it be that these substances, although very effective against histamine, and this in very different degree, also have in higher dosage an antagonistic effect, and this in equal degree, against some other substance involved in anaphylaxis?

There is general agreement that histamine is only one of a number of substances released as the result of an antigen-antibody reaction. We know that heparin is set free, but not much is known concerning other substances which may be liberated

Dreisbach (22) demonstrated that Pyrribenzamine and Benadryl fail to inhibit the Arthus phenomenon in rabbits, an observation confirmed by Fischel (23) for Pyrribenzamine. Dreisbach presumed that the central and the peripheral sensory depressions of these agents are responsible for such antiallergic effects as have been observed. The anesthetic effect of antihistamine substances has been (24) the object of much interest in the recent past (24). Code and co-workers found that this anesthetic effect was independent of the inhibitory action of the drugs on skin wheals. Mayer and co-workers (16), using a 2% Procain spray, could not protect guinea pigs against histamine death, but a 2% Pyrribenzamine spray had given this protection. More elaborate studies of this aspect of the question are needed.

The evidence in regard to the ability to shock guinea pigs for a second time after they have survived anaphylactic shock because of protection by an antihistamine substance is conflicting. This question is important, because it may shed light on the mechanism of protection. If the compounds interfere with the antigen-antibody reaction, as is suggested by our observations and those of others, the whole concept of the action of these drugs deserves new examination.

SUMMARY

- 1 The minimal dose that gave guinea pigs 100% protection against one lethal dose of histamine, administered intracardially, was deter-

mined for nine antihistamine compounds, administered subcutaneously. The effective dose varied from 0.02 mg/kg for Neo-Antergan to 1.5 mg/kg for Antistine.

2 Protection against anaphylactic shock required much higher dosage than protection against histamine intoxication. The protective dose of the various compounds was almost equal, with the exception of Antistine.

3 Of 19 animals that had been protected against anaphylactic shock, 17 could be shocked 36 hours later.

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COMPARATIVE STUDY OF ANTIHISTAMINE SUBSTANCES¹

III CLINICAL OBSERVATIONS

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Therapy in allergic conditions has advanced immensely since, four decades ago, Dale laid the cornerstone of the present efforts in "antihistamine therapy" In adrenalin, butanefrine, ephedrine, neosynephrine, etc. we possess powerful drugs for symptomatic treatment For bronchial asthma, the introduction of aminophyllin, of oxygen-helium inhalations, and recently of massive doses of penicillin (Gay and Marriot) (1) has supplied additional weapons Diagnosis by careful analysis of the history and by adequate skin tests will allow elimination of the responsible allergen from the environment, or desensitization treatment, or both, with excellent results in many cases

Nevertheless, the need for further progress is still widely felt The sympathicomimetic substances mentioned have unpleasant side effects, and in severe cases they are effective only parenterally Discovery of the provoking agent or mechanism is not possible in every instance, and desensitization is not always feasible or successful Therefore, after the failure of histaminase and histamine azoproteine, the introduction of compounds that counteracted histamine effects and anaphylactic death in animals was greeted with high hopes

The first reports (1942) on the initial French preparation, Antergan, were conflicting, but as a whole they were encouraging When the first American drug, Benadryl, became available, we used it in 101 cases The results were inconsistent, the side effects very frequent and disturbing Other compounds were offered to us in rapid succession In preliminary screening, we eliminated five because of frequent side reactions or lack of clinical effect Eight preparations were finally

¹ This investigation has been aided by grants from G D Searle and Company, Eli Lilly and Company and the Houston Endowment, Incorporated

² From the Protein Clinic of the Department of Medicine, The Johns Hopkins Hospital

chosen for comparative clinical study, five of these receiving more extensive trial. They were used in dispensary and private patients. As mentioned in the introduction to this entire study, each drug was assigned to at least two members of our group³.

The evaluation of new drugs is particularly difficult in allergic conditions because of the psychoneurotic factor in many cases, because of the fluctuating pollen content of the air during the pollen season or variable degrees of exposure to other agents, because of the unpredictable, sometimes self-limited, character of certain conditions, e g acute urticaria. Placebos were substituted extensively for the drugs. A considerable number of patients, particularly in the dispensary, had to be eliminated from the study on the basis of their placebo response.

During a twelve months period, ending on December 1, 1947, 686 patients were treated with the drugs. This figure, however, appears only in the tabulation of the side effects. For comparison of the results in the various conditions, we disregarded very small groups and limited the tabulation to drugs given to a sufficient number of patients suffering from the same condition.

Where to draw the line in considering a patient benefited will always be a matter of somewhat arbitrary decision. We registered our cases under the following groups: No Improvement, Improved 25%, 50%, 75%, 100%. For the final evaluation, we regarded patients who showed 50% or more relief from their symptoms as benefited.

The dosage of the drugs, with two exceptions, was 50 mg every four to six hours. In preliminary trials it became apparent that this dose was too small for Antistine. Originally, this drug had been used in 100 mg doses in Switzerland, accordingly, we gave it in this dosage in all cases listed. Hydryllin contains 100 mg aminophyllina and 25 mg diphenhydramine base. On the basis of its antihistamine content, 25 mg would seem to be the effective dose in this combination.

RESULTS

(a) *Seasonal and perennial allergic rhinitis* (Table 1) There was no evidence of a significant difference in the results between seasonal, or

³ For description of the drugs used, see I. Introduction and Dale Experiments Bull Johns Hopkins Hospital, 83: 331, 1948,

pollen, rhinitis and perennial rhinitis, when the latter was definitely of allergic origin. The two types were therefore considered together. Of 428 cases, 60% to 76% were benefited. The best results were obtained with Pyribenzamine, followed by Hydryllin, Antistine, and Neo-Antergan. Relieved were the itching of eye and nose, the watery discharge and the sneezing paroxysms. Nasal obstruction was rarely influenced. Patients with perennial vasomotor rhinitis of the non-allergic type, characterized by negative personal and family history of allergy, by negative skin tests, and by psychoneurotic background were not tabulated because of their small number. They could not be

TABLE 1

Effect of various antihistamine compounds on seasonal and perennial allergic rhinitis

DRUG	NUMBER OF PATIENTS	% BENEFITED
Antistine	43	70
Hydryllin	97	73
1721 (Searle)	23	65
1913 (Searle)	19	68
Histadyl	63	63
Chlorothen	30	60
Pyribenzamine	51	76
Neo-Antergan	102	70
Total	428	68

helped by any antihistamine drug, those who seemed to be temporarily improved had the same result with placebos.

(b) *Bronchial Asthma* (Table 2) 96 cases are listed. The number is small, for it soon became obvious that severe cases of asthma did not respond to the drugs, and had to receive adrenalin and aminophyllin for relief. We had, therefore, to restrict the administration of the drugs in asthma in order to avoid needless prolongation of the attacks. Neo-Antergan and Hydryllin appeared to be most effective in these small series. On the basis of closer analysis and extended experience, the figures of Table 2 have to be qualified by the statement that only mild cases of wheezing could be relieved. It was particularly disappointing that ragweed-allergy victims whose nasal symptoms had responded well to one of the drugs during last year's ragweed season

contracted severe asthma in unusually large numbers at the end of the season and failed to obtain any help from the drugs at this time

(c) *Urticaria and angioneurotic edema* (Table 3) In 53 cases, relief varied from 60% to 89% Antistine appears here on top of the list The number of cases is too small to warrant conclusions, except the statement that the highest degree of relief can be obtained in this condition The response was rapid and complete, especially in urticaria caused by penicillin

TABLE 2

Effect of various antihistamine compounds on bronchial asthma

DRUG	NUMBER OF PATIENTS	% BENEFITED
Antistine	18	33
Hydriyllin	26	46
Histadyl	10	40
Pyribenzamine	15	33
Neo-Antergan	27	48
Total	96	40

TABLE 3

Effect of various antihistamine compounds on urticaria and angioneurotic edema

DRUG	NUMBER OF PATIENTS	% BENEFITED
Antistine	19	89
Hydriyllin	10	80
Histadyl	14	79
Neo-Antergan	10	60
Total	53	77

(d) *Dermatitis and pruritic conditions of various etiology* (Table 4) Of 56 cases, Antistine and Histadyl benefited in 72% and 70%, respectively Relieved was the itching, but not the skin disease itself Cessation of scratching naturally resulted in improvement of such skin lesions as were due to superimposed trauma There was no evidence that the results in atopic and contact dermatitis differ in this respect Pruritus an responded excellently in most of the cases, but in a few there was complete failure

(e) *Total results in all conditions treated* (Table 5) If we limit our evaluation to the five drugs with which large groups of patients were treated, we find that an average of 65.4% of all cases treated was helped by the drugs. The incidence of beneficial results ranged from 62% with Histadyl to 69% with Pyribenzamine.

These figures suggest that there is no fundamental difference in the effectiveness of the various drugs. This impression is confirmed when

TABLE 4

Effect of various antihistamine compounds on dermatitis and pruritic conditions of various etiology

DRUG	NUMBER OF PATIENTS	% BENEFITED
Antistine	29	72
Histadyl	27	70
Total	56	71

TABLE 5

Effect of various antihistamine compounds on all conditions treated
(Summary)

DRUG	NUMBER OF PATIENTS	% BENEFITED
Antistine	110	67
Hydryllin	142	65
Histadyl	119	62
Pyribenzamine	71	69
Neo-Antergan	147	64
Total	589	65.4

we consider those patients who have been treated with different drugs in succession.

The change from one drug to another produced results better than, equal to, or worse than those experienced before. There was no rule, in the sense that one drug was always more effective or less effective than another drug. Of two patients with the same condition, one might benefit from drug No. 1 but not from drug No. 2, whereas, the reverse might be true for the second patient. Table 6 gives a particular illustration, indicating the results in hay fever patients when Hydryllin was replaced on 65 occasions by other drugs.

(f) *Side effects* (Table 7) The frequency of side effects in the 686 patients treated with the eight different drugs varied from 13% with Antistine to 42% with 1913 (Searle) The frequency of side effects was also high with 1721 (Searle) and Hydryllin The second lowest incidence of side effects was noted with Histadyl 119 of the 686 cases

TABLE 6

Effect on hay fever of Hydryllin compared with other compounds in the same patients

OTHER DRUG	BETTER THAN HYDRYLLIN	EQUAL TO HYDRYLLIN	WORSE THAN HYDRYLLIN	TOTAL
Antistine	—	1	1	2
Benadryl	4	8	6	18
1721 (Searle)	3	3	—	6
Histadyl	3	4	5	12
Chlorothen	1	1	—	2
Pyribenzamine	7	7	7	21
Neo-Antergan	2	1	1	4
Total	20	25	20	65

TABLE 7

Side effects of various antihistamine compounds

DRUG	NUMBER OF PATIENTS	% TOTAL SIDE EFFECTS	% SEVERE SIDE EFFECTS
Antistine	110	13	5
Hydryllin	142	36	18
1721 (Searle)	36	36	25
1913 (Searle)	26	42	6
Histadyl	119	19	6
Chlorothen	35	23	0
Pyribenzamine	71	33	25
Neo-Antergan	147	27	3
Total	686		

complained of drowsiness Other side effects were dizziness, weakness and fatigue, headache, nervousness, tremor, apprehension, tachycardia, anorexia, nausea, abdominal pain, diarrhea, dryness of mouth, blurred vision, dysuria, urinary frequency Severity of side effects was more frequent with Pyribenzamine, 1721, and Hydryllin than with the other drugs The individuality of response, mentioned in the discussion of

total results, holds true also for occurrence of side effects. Many a patient who could not tolerate one drug could change to another drug which caused side effects in other patients but not in him.

DISCUSSION

Our results are in basic agreement with those of most other observers. Arbesman and co-workers, (2) e g, reported improvement in 75% of their patients with allergic rhinitis when treated with Pyrribenzamine. In bronchial asthma these authors found 48% improved by this drug, a figure considerably higher than ours. Bernstein and co-workers (3) found that of the patients with nonseasonal and seasonal allergic rhinitis, about 76% were improved by Pyrribenzamine and about 65% by Neo-Antergan, in asthma, only 28% were benefited by Pyrribenzamine and only 20% by Neo-Antergan. Fuchs and co-workers (4) failed to find any difference in relief from hay fever between a group treated with ragweed extract alone and a group treated with ragweed extract plus Pyrribenzamine. However, they employed a fixed dose of 150 mg daily in all patients, regardless of severity of their symptoms.

The Committee on Therapy of the American Academy of Allergy (5) has released a report based on 1570 cases treated with Hydryllin. This report shows 'results less favorable in hay fever and vasomotor rhinitis but more favorable in asthma than our study shows. As the figures have been compiled from the observations of 44 investigators, it is doubtful that they are based on identical methods of evaluation.

The same report gives figures of the results with Antistine. Our observations, as well as those of others, disagree with these figures. Only 24% good and 13% fair results were encountered in the Committee's 114 cases of hay fever. Walton (6) found improvement in 73% of his 44 patients with perennial rhinitis. He treated only 4 cases of seasonal allergic rhinitis, all of which improved. 80% of his urticaria patients improved. Britton (7) reported benefit from Antistine in 76% of his allergic patients. Hughes (8) had good results with Antistine in 59% of 32 cases with various allergic conditions.

Feinberg and Bernstein (9) studied the effect of Histadyl. 70% of patients with seasonal hay fever, but only 40% of those with non-seasonal vasomotor (allergic?) rhinitis, were relieved. In urticaria and dermatitis, their results were somewhat less favorable than ours.

The incidence of side effects depends upon the dosage employed Britton, *et al.*, noticed side effects with Antistine in 37% of the patients, 13% severe, obviously because he gave 200 mg of the drug four times a day With the same drug, Walton had 25% side effects, less than 6% severe, Hughes reported reactions in only 18%, almost all mild When in our study we correlated the dosage level with the incidence of side reactions, it became apparent that the critical dosage of Antistine (where the reaction curves swing up) was 400 mg daily

This critical dosage was 100 mg for Hydryllin and 200 mg for Histadyl Beginning with these doses, the frequency of reactions increased in our cases

Several cases of dermatitis due to Pyribenzamine have been reported (Arbesman and co-workers (2), Epstein (10), Harris and Shure (11)) We have not seen such reaction to any of the drugs Exacerbation of asthma from use of Pyribenzamine has been noticed by Henderson and Rose (12) A similar observation had been made in several instances with Benadryl (Waldbott (13), Levin (14))

CONCLUSIONS

The results of our study of the so-called antihistamine substances demonstrate that no parallelism exists between their effectiveness against histamine in guinea pigs and their effectiveness against human allergy The wide differences in potency found in histamine experiments are not found in clinical use However, there may be a relationship between clinical effect and effect in animal anaphylaxis Only Antistine required a much higher dose for protection against anaphylactic death than did the other drugs The same drug is effective in man only when twice as high a dose is used as is necessary with the other compounds These other compounds gave a very similar degree of protection in animal anaphylaxis and a very similar incidence of benefit in clinical use Such differences between the drugs as have been seen in both uses are hardly significant

In selecting a drug for the treatment of an allergic condition, one should consider its side effects as well as its percentage of good results If it is imperative to avoid drowsiness, as in patients who have to operate engines of any kind, a drug like Histadyl or, when it has become available, Antistine is indicated Where a sedative effect is

not only non-hazardous but even desirable, a preparation like Pyribenzamine, Hydryllin or Neo-Antergan is preferable, particularly at bedtime. It is fortunate that we are able to choose among several preparations and to change from one to another whenever necessary.

The indications for using the compounds are clear. They cannot replace the diligent search for the etiological factor in allergic conditions and its elimination, if found. While this search is being conducted and in case of failure, these compounds are very helpful adjuvants in allergic rhinitis and urticaria. Their effectiveness in relieving the itching in many conditions makes them particularly valuable, in fact, in this respect they occupy a singular place in our armamentarium. The drugs are not indicated in bronchial asthma, except in the mildest cases. Their administration during an asthmatic attack will unnecessarily prolong the suffering of the patient. All critical observers have emphasized the poor results of these drugs in asthma, a fact that contrasts strangely with their effectiveness against the bronchoconstrictor action of histamine in guinea pigs.

Even in conditions in which we see the highest incidence of beneficial results, complete failures sometimes occur. The nature of such cases deserves closer analysis. The fate of the drugs during their passage through the gastro-intestinal tract should be determined.

Further concentrated efforts are necessary in order better to understand the action of "antihistamine substances," and to eliminate their side effects.

SUMMARY

1 Eight different antihistamine compounds were tested in various allergic conditions.

2 Of 589 cases, 65.4% were benefited. The best results were obtained in urticaria, followed by allergic rhinitis and dermatitis. In bronchial asthma, only the mildest cases were benefited.

3 There were no pronounced differences in the effectiveness of the drugs. The response to the drugs differed from patient to patient. However, a drug which failed or caused severe side effects was frequently replaceable by another drug with good success.

4 Side effects were least frequent with Antistine, although its effective dose was twice that of the other drugs.

5 The differences in potency of the drugs against histamine in guinea pigs were not paralleled by similar differences in clinical results

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THE CULTURAL DIFFERENTIATION OF PARACOLON BACILLI

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The identification of paracolon bacilli and the differentiation of these organisms from *Salmonella*, *Shigella*, *Proteus*, and other Gram negative bacilli which fail to ferment lactose, present one of the most difficult problems in diagnostic bacteriology. This problem was emphasized and discussed in a previous report on the relation of paracolon bacilli to urinary tract infections (Schaub, 1946). The investigation to be reported here was undertaken with the hope of finding a method for the differentiation of paracolon bacilli from culturally similar *Enterobacteriaceae* and for the classification of the paracolon bacilli.

Cultural studies of paracolon bacilli by Stuart, Wheeler, Rustigian, and Zimmerman (1943) resulted in the now generally accepted practice of differentiating these Gram negative bacilli into paracolon *Escherichia*, paracolon *Aerobacter* and paracolon intermediate on the basis of their IMViC* formulae. This method of differentiation parallels the classification by Parr (1936) and Stuart, Griffin and Baker (1938), of the typical coliform bacilli into *Escherichia*, *Aerobacter* and intermediates on the same basis, that is, by their indol, methyl red and Voges-Proskauer reactions and utilization of citrate as the sole source of carbon. Borman, Stuart and Wheeler (1944) suggested the generic name *Paracolobactrum* for the paracolon bacilli and the division of this genus into *Paracolobactrum aerogenoides*, *Paracolobactrum intermedium* and *Paracolobactrum coliforme* on the basis of acetyl methyl carbinol production and utilization of citric acid as sole carbon source. Michael and Harris (1945) also concluded, as the result of a cultural study of a group of paracolon bacilli, that, other than the IMViC reactions, no biochemical properties could be considered characteristic of any one

*IMViC is a mnemonic formula proposed by Parr (1938) and expresses the results of the indol, methyl red and Voges-Proskauer reactions and the utilization of citrate as the sole carbon source.

group of paracolon bacilli and that there was no cultural basis for classification except by determination of IMViC formula. Certain types or sub-groups of paracolon bacilli have been the subject of special biochemical and serological investigation (Peluffo, Edwards and Bruner, 1942, Edwards, Cherry and Bruner, 1943, Edwards, 1945, Hinshaw and McNeil, 1945, Barnes and Cherry, 1946, Stuart and Rustigian, 1943, Stuart, Wheeler and McGann, 1946, Galton, Hess and Collins, 1947). However, these types have been selected primarily on the basis of their probable pathogenicity for man and animals, and these reports have not contributed to the differentiation of the paracolon group as a whole.

In our laboratory fifty-six strains of paracolon bacilli, with but one exception from human sources, have been studied. Sixteen of these cultures were received from Dr L W Parr of George Washington University, twelve from Dr C A Perry and A A Hajna of the Maryland State Department of Health, four strains from Dr R V Stone of the Los Angeles Health Department and one from Mr T C Buck, Baltimore City Health Department. The remaining twenty-three strains were recently isolated in laboratories of the Johns Hopkins Hospital from a variety of clinical material, for the most part urine and stool cultures. One hundred control cultures, consisting of a number of strains each of *Escherichia*, *Aerobacter*, intermediate coliform bacilli, *Salmonella*, *Shigella*, *Eberithella*, *Proteus*, *Pseudomonas*, *Serratia*, *Alkaligenes*, *Vibrio* and a few miscellaneous Gram negative bacilli were also included in the series.

The usual biochemical tests used in the differentiation of Gram negative bacilli were employed in the preliminary study of these cultures. These were 1) the fermentation of lactose, dextrose, sucrose, xylose and mannitol, 2) the production of indol, the methyl red and Voges-Proskauer reactions and the utilization of citrate, that is, determination of IMViC formula, and 3) reduction of nitrates, production of hydrogen sulfide, utilization of sodium malonate (Leifson, 1933) and liquefaction of gelatin. This cultural study yielded the anticipated results, that the paracolon bacilli could be classified as paracolon *Escherichia*, paracolon *Aerobacter*, and paracolon intermediate on the basis of their IMViC formula, and that these biochemical reactions would not allow rapid differentiation of the paracolon bacilli from *Salmonella*, *Shigella*, *Proteus*, and other non-coliform organisms.

A number of special media recently suggested for the differentiation of paracolon bacilli from *Salmonella*, *Shigella* and *Proteus* were then studied. These included the multiple sugar medium and 10% lactose agar slants of Chilton and Fulton (1946) and Christensen's urea agar (1946). In the multiple sugar medium, which contains adonitol, aesculin, salicin and sucrose and in which fermentation indicates paracolon bacilli as differentiated from *Salmonella*, which fail to ferment any of these carbohydrates, our results closely approach those of Chilton and Fulton, 33% of the paracolon bacilli in our series giving a positive reaction. However, the low percentage of paracolons which give fermentation in this medium does not recommend it for the differentiation of paracolon bacilli from the non-coliform Gram negative bacilli which give a negative reaction. The agar slants containing 10% lactose were also found to be of little value, since only 21% of our paracolon bacilli showed fermentation in 24 hours on this medium, while 20 strains of supposedly lactose-negative Gram negative bacilli in the control series, including strains of *Eberthella*, *Shigella* and *Salmonella*, gave an acid reaction.

Christensen's urea agar indicated urease production by 29% of the paracolon bacilli studied, thus differentiating these strains from the urease-negative *Salmonella*, *Shigella* and *Eberthella*. The delayed reaction of the paracolon bacilli as contrasted with the rapid urease production by *Proteus* was useful in differentiating these organisms, as pointed out by Christensen. However, the small percentage of paracolon bacilli found to produce urease detracts greatly from the value of the urea agar for the differentiation of paracolon bacilli from other *Enterobacteriaceae*.

Therefore, since neither the usual methods for the biochemical differentiation of Gram negative bacilli nor the new media suggested by Chilton and Fulton and by Christensen seemed to solve the problem of identifying paracolon bacilli or rapidly differentiating them from *Salmonella* or *Shigella* and other non-coliform Gram negative bacilli, the following investigation was undertaken.

It is well known that the growth of the typical coliform bacilli, *Escherichia*, *Aerobacter*, and the intermediate group, is inhibited on certain media designed for the isolation of the intestinal pathogens and for the inhibition of the normal intestinal Gram negative bacilli. It seemed possible that the paracolon bacilli also might be selectively

inhibited on such media, since they are generally regarded as atypical or aberrant forms of normal coliform organisms. Therefore, the growth of the paracolon bacilli in our series, as well as that of all of the control cultures, was studied on such media (Schaub, 1947).

Inhibition of growth on Baltimore Biological Laboratory desoxycholate-citrate and desoxycholate-citrate-lactose-sucrose agar and on Difco-Salmonella-Shigella (S S) agar was first determined. In preliminary trials, S S agar was found to give the best results, inhibition of growth on desoxycholate-citrate-lactose-sucrose agar being almost as satisfactory. S S agar was therefore selected as the test medium for the original study. However, we were unable to obtain information as to the composition of Difco bile salts no. 3, which is one of the active ingredients of S S agar, and since, therefore, the formula of S S agar is not completely known, and since it is over complicated for our purpose, the development of a suitable medium of known composition was undertaken. This investigation has led to the development of a simple coliform inhibiting medium with the following formula:

Beef extract	0.5%
Sodium citrate	1%
Sodium desoxycholate	0.25%
Sodium thiosulfate	1%
Trypticase	0.5%
Agar	1.4%

pH 7.1

This coliform inhibiting medium, which will be referred to hereafter in this report as C I agar, has been found to give the same results as were obtained with Difco S S agar, that is, excellent inhibition of the typical coliform bacilli and closely related paracolons, while supporting the growth of the control cultures and of certain paracolon bacilli, as will be described.

In this investigation, C I and S S agar have been used both as agar plates and slants with equally good results. S S agar was prepared from the dehydrated, commercial product as directed, without sterilization, and distributed aseptically into sterile petri dishes or into sterile test tubes and slanted. C I agar was prepared according to the formula given above with a minimum of heating and also distributed aseptically into sterile petri dishes or into sterile test tubes and slanted.

In the aseptic tubing of both these media only simple precautions need be taken since the media will not support the growth of common contaminants

In testing simultaneously a large number of cultures, both C I and S S agar have been used in petri dishes. A plate may be divided into as many as twelve segments and each segment streaked lightly with a proper dilution of the culture as will be described. In testing single cultures, the media tubed and slanted is preferred, since a relatively small surface area gives better evidence of the presence or absence of inhibition than if an entire plate is inoculated with a diluted culture. In using S S agar, the medium in slants was found to have the added advantage of demonstrating hydrogen sulfide production as satisfactorily as the lead acetate medium used for this purpose, the medium at the base of the slant becoming markedly blackened when the surface of the slant is streaked with a hydrogen sulfide producing organism. C I agar gives only slight evidence of hydrogen sulfide, and this irregularly and only after 48-72 hours incubation.

On both the C I and S S agar, inoculation with undiluted broth cultures was found to give unreliable results. However, if diluted cultures are used, consistently satisfactory, reproducible inhibition of the typical and aberrant coliform bacilli was obtained. The dilution factor operates within very broad limits, it having been demonstrated that a dilution of one loopful of a 24-hour broth culture in broth or saline in amounts varying from 2 to 15 cc gives equally good results. In this study we have consistently used a well shaken dilution of a loopful of a 24-hour broth culture in 10 cc of sterile isotonic salt solution. It has also been demonstrated that comparable results can be obtained by fishing directly from a colony on desoxycholate agar to 10 cc of sterile salt solution, using the saline suspension for the inoculation of the test medium.

Using the techniques described above, inhibition of the coliform and closely related paracolon bacilli is usually complete. Occasionally one or two colonies of these organisms are found on either of the coliform inhibiting media. Inhibition of growth, however, is definitely indicated in such instances, as the cultures in which no inhibition occurs give numerous colonies when a diluted inoculum is used. It should be emphasized that results must be read after 18-24 hours incu-

bation After longer periods of incubation growth may occur in cultures which were completely inhibited in 24 hours

Studying the growth of diluted and undiluted cultures on both C I and S S agar as described, it was found that the growth of the typical coliform bacilli, *Escherichia*, *Aerobacter* and the intermediates, was inhibited, while a majority of the control cultures, including *Salmonella*, most *Shigella*, *Eberthella*, *Proteus* and *Pseudomonas*, grew well. The notable exceptions in the control series were several old stock strains of *Shigella dysenteriae*, which were inhibited irregularly on both media, and all three strains of *Proteus morgani*, which were completely inhibited on C I agar while growing well from a diluted inoculum on S.S agar. Also inhibited on both C I and S S agar were all strains of *Serratia marcescens*, *Alkaligenes faecalis*, *Bacterium bronchisepticum* and *Vibrio comma*, *Vibrio metchnikovi* and *Vibrio proteus*. However, since these latter organisms may be readily differentiated from paracolon bacilli, their failure to grow on C I and S S agar is not considered to detract from the value of these media as used for the identification of paracolon bacilli.

When the paracolon bacilli in our collection were studied on these media, it was found that the growth of a large number was inhibited, while others grew equally as well as the control cultures. When the fact of growth or inhibition on coliform inhibiting media was correlated with the results of the cultural study, all of the paracolon bacilli in our series were found to fall into one of four groups, which for the sake of convenience have been designated Groups I, II, III, and IV (Table I).

The paracolon bacilli whose growth was inhibited on C I and S S agar are included in Group I. In this respect, that is, inhibition on the test media, and in other cultural characteristics as well, these paracolon bacilli resemble the coliform bacilli, differing from the typical coliforms only in their failure to ferment lactose promptly with acid and gas. Stuart, Mickle and Borman (1940) have suggested the term "aberrant coliform" for the slow-lactose-fermenting coliform organisms, and this term may well be applied to these Group I paracolon bacilli. Stuart and his associates (*ibid*) suggested that the aberrant coliforms be

TABLE I
Cultural Differentiation of *Paracolon Bacilli*

ORGANISM	MOTILITY	LACTOSE	DEX- TROSE	SUCROSE	XYLOSE	MAN NITOL	INDOL	METHYL RED	VOGES PROS- KAUER	CITRATE	MALON- ATE	NITRATES	HYDRO- GEN SULFIDE	UREASE	GROWTH OF DILUTED CUL- TURE ON CI OR S S AGAR
<i>Group I paracolon bacilli</i> <i>Paracolon Escherichia</i>	+/-	late Ag, A only or nega- tive	Ag/A	Ag/A/-	Ag/A	Ag/A	+	+	-	-	-	+	-	-	-
<i>Paracolon Aerobacter</i>	+/-	late Ag, A only or nega- tive	Ag/A	Ag/A	Ag/A	Ag/A	-	-	+	+	+	+	-	+/- ³	-
<i>Paracolon intermedia</i>	+/-	late Ag, A only or nega- tive	Ag/A	Ag/A/-	Ag/A	Ag/A	Combination other than those above			+	+/-	+	-	+/-	-
<i>Group II paracolon bacilli</i> ¹	+	late Ag	Ag	-/Ag	Ag	Ag	-	+	-	+	-/+	+	+	+/-	+
<i>Group III paracolon bacilli</i>	+	Negative	A ²	A late	-	-	+	+	-	+	-	+	+ ^w	-	+
<i>Group IV paracolon bacilli</i>	+	late A or negative	A, sl gas	-/A, sl gas	Ag/A	Ag/A	-	+	+	+ ^w	+	+	-	-	+

Ag = acid and gas, A = acid only, sl = slight, w = weak, +/- = some strain positive, some negative

¹ For differentiation of the subgroups of Group II paracolon bacilli, see Table III

² Some strains produce a bubble of gas in dextrose Durham tubes

³ + = slight urease production in 24 hours, increasing on further incubation, on Christensen's urea agar

classified by their reaction in lactose Durham tubes and described the following groups

- a) late-lactose-fermenting, micro-aerogenic, which fail to produce acid and 20% or more gas in lactose broth in 48 hours
- b) anaerogenic, which ferment lactose in 24 hours or longer with the production of acid but no gas
- c) papillae-forming strains, which ferment lactose slowly with acid and gas and which, on desoxycholate or eosin-methylene-blue agar, produce colored secondary colonies, transplants from which ferment lactose in 24 hours. These include the organisms generally known as *Escherichia coli-mutabile* (Neisser, 1906, Massine, 1907, Parr, 1939) but the group is not limited to paracolon *Escherichia*
- d) strains which fail completely to ferment lactose

The paracolon bacilli of Group I of our series may be classified by their fermentation of lactose in this manner, and examples of each fermentative type have been found in the cultures studied. It is interesting to note that with but one exception, all the strains of paracolon *Escherichia* which fermented lactose slowly produced papillae-forming colonies on desoxycholate agar, either readily on first isolation or after a number of subcultures. Only one strain each of paracolon *Aerobacter* and paracolon intermediate were found to produce such secondary colonies.

As has been previously mentioned, Stuart, Wheeler, Rustigian and Zimmerman (1943) and Borman, Stuart and Wheeler (1944) introduced the classification of paracolon bacilli as paracolon *Escherichia*, paracolon *Aerobacter* and paracolon intermediate by IMViC formula. As shown in Table II, the Group I paracolon bacilli, those whose growth is inhibited on C I and S S agar, may be thus differentiated by their indol, methyl red, Voges-Proskauer and citrate reactions. As far as other biochemical reactions are concerned, there is no correlation between the results in Chilton and Fulton's multiple sugar (Aass) medium and differentiation by IMViC formula, while on Christensen's urea agar paracolon *Escherichia* were uniformly negative and paracolon *Aerobacter* and paracolon intermediates were found to be less than 50% positive. Other cultural characteristics are shown in Table I.

The strains of paracolon bacilli comprising Group I were isolated

from both normal and pathologic stool specimens, from urine, from blood and body fluid cultures taken at autopsy, and from ear cultures. The percentage occurrence of this and the other groups of paracolon bacilli in our series is not significant, since they do not represent a continuous series, and the strains received from Dr. Parr, Dr. Perry and Mr. Hajna, and Dr. Stone represent selected strains from their collections. However, since efforts to obtain additional freshly isolated strains of Group II, III and IV paracolons have been unsuccessful, and since Group I cultures are encountered with great frequency in the routine laboratories of the Johns Hopkins Hospital, it is evident that the aberrant coliform bacilli of Group I are by far the most common paracolon bacilli encountered in clinical material from human sources. Using inhibition on C I or S S agar as a screen test, these organisms may be rapidly differentiated from non-coliform bacilli, such as *Salmonella*, *Shigella* and *Proteus*, which they may resemble culturally.

Thirty seven of the paracolon organisms in our series failed to grow on C I or S S agar, and are so included in Group I just described. The remaining nineteen strains grew well under the test conditions. If classification by IMViC formula alone was applied, without reference to growth on the test media, then these organisms would be indiscriminately classified as paracolon intermediates. But if they are separated from the aberrant coliform bacilli of Group I by the fact of their growth on coliform inhibiting media, then these organisms fall into three well defined groups, Group II, Group III and Group IV.

The outstanding common characteristic of Group II paracolon bacilli, as shown in Table I, is their production of hydrogen sulfide. They are all motile Gram negative bacilli which ferment lactose slowly, in from 2-10 days, with the production of acid and slight gas, serial transfer in lactose broth resulting in fermentation in 24 hours. Dextrose and mannitol are fermented promptly with acid and gas. An IMViC formula of $- + - +$ is common to the group.

Other biochemical characteristics of the Group II paracolon bacilli in our collection have shown these organisms to fall into three well defined cultural sub-groups, as shown in Table II. The organisms of sub-group A are characterized by the production of urease as demonstrated on Christensen's urea agar, though the reaction is weak and delayed as compared with that of *Proteus*. They also may be readily

recognized by the presence of a marked and distinctive odor resembling that of spoiled cabbage. These strains correspond to the description published by Barnes and Cherry (1946) of a group of paracolon bacilli isolated from a small outbreak of gastroenteritis in Bethesda, Maryland. Dr P R Edwards has kindly studied the strains comprising Group II-A, and has classified them (Edwards, 1947) as belonging to what he refers to as the "Bethesda Group", which group includes the strains from gastroenteritis described by Barnes and Cherry. The strains of sub-group A in our series were also isolated from stool specimens from patients with gastroenteritis.

Sub-group B of Group II paracolon bacilli differs from sub-Group A in fermenting sucrose and in producing acid and gas in Chilton and Fulton's multiple sugar medium, probably due to fermentation of the sucrose in this medium. These organisms also possess the distinctive characteristic of growing on nutrient agar containing 1/5000 potassium tellurite. The only other Gram negative bacilli found to grow on tellurite agar of this concentration are *Proteus mirabilis*, *P. vulgaris*, *P. morgani* and *Pseudomonas aeruginosa*. It is possible that the sucrose-positive, hydrogen-sulfide-positive, urease-producing paracolon bacilli reported by Christensen (1947) may be of this group.

Sub-group C of Group II paracolon bacilli may be differentiated from Group II-A and Group II-B by failure to produce urease, by utilization of sodium malonate, by lack of the distinctive cabbage odor and by slow liquefaction of gelatin. Culturally these organisms correspond to descriptions published by Peluffo, Edwards and Bruner (1942), Edwards, Cherry and Bruner (1943) and Hinshaw and McNeil (1945) of paracolon bacilli isolated from warm and cold blooded animals and turkeys, which organisms are serologically related to the genus *Salmonella*. In only one instance, Edwards (1945), has a paracolon bacillus of this group been reported from a human infection. The one strain of Group II-C in our collection was received indirectly from Dr Hinshaw and was isolated from a rattlesnake.

These hydrogen sulfide producing paracolon bacilli of Group II may, on superficial cultural examination, be confused with *Salmonella* or *Proteus*, but they may be readily differentiated culturally as shown in Table II. *Proteus* may be eliminated by the use of either Rustigian and Stuart's (1941) or Christensen's (1946) urea media. Sub-groups

A and B may be rapidly differentiated from *Salmonella* by urease production on Christensen's urea agar, less specifically by the distinctive cabbage odor, and eventually by their late fermentation of lactose. Sub-group C can be differentiated from *Salmonella* by utilization of sodium malonate and by the slow lactose fermentation and delayed gelatin liquefaction of these paracolon bacilli.

Group III includes a well defined group of anaerogenic paracolon bacilli which grow well on SS and CI agar. As shown in Table I, they fail to ferment lactose, xylose and mannitol while fermenting dextrose with the production of acid and usually no gas, although occasionally a small bubble of gas may be formed. Sucrose is fermented slowly, in from 4-6 days, with acid only. Indol is produced and citrate utilized (IMViC + + - +), while the reaction on Christensen's urea agar is negative. In lead acetate medium, hydrogen sulfide is produced, but the reaction is weak.

These Group III paracolon bacilli, the four strains of which in our collection were isolated from patients with intestinal infections, are culturally identical with paracolon bacilli Type 29911 as described by Stuart, Wheeler and McGann (1946). Stuart and his associates concluded, after an extensive study of their Type 29911 cultures, that they appear to be an intermediate group between *Proteus* and *Shigella*, a concept borne out by our results. Culturally, Group III paracolon bacilli closely resemble *Proteus rettgeri*, but they may be easily differentiated by the strong urease production of the latter.

The biochemical characteristics of Group IV paracolon bacilli are shown in Table I. Like Groups II and III, their growth is not inhibited on either of the coliform inhibiting media, so that they may be readily differentiated from the commonly occurring Group I organisms. The outstanding cultural characteristic of Group IV paracolon bacilli is their utilization of sodium malonate as the sole source of carbon. The ability to utilize sodium malonate or malonic acid is a valuable differential characteristic, since it is shared by a relatively few Gram negative bacilli, namely *Aerobacter* and Group I paracolon *Aerobacter*, some strains of intermediate coliform bacilli and Group I paracolon intermediates, Group II-C and Group IV paracolon bacilli and also *Pseudomonas aeruginosa*. Of these malonate-positive organisms, only *Pseudomonas* and Group II-C and Group IV paracolon bacilli will grow

on coliform inhibiting media, and the malonate-positive paracolons may be readily differentiated as shown in Tables I and II

TABLE II
Differentiation of Group II Paracolon Bacilli

ORGANISMS	HYDROGEN SULFIDE	LACTOSE	SUCROSE	UREASE ¹	GELATIN	MALONATE	CAB BAGE ODOR	GROWTH ON AGAR CONTAINING 1/5000 POTASSIUM TELLURITE
Group II—A paracolon bacilli	+	late AG	—	+	—	—	+	—
Group II—B paracolon bacilli	+	late AG	AG	+	—	—	+	+
Group II—C paracolon bacilli	+	late AG	—	—	+ slow	+	—	—
<i>Salmonella</i>	+/-	—	—	—	—	—	—	—
<i>Proteus vulgaris mirabilis</i>	+	—	AG 1-7 days	++	+	—	—	+
<i>Proteus morganii</i>	+	—	—	++	—	—	—	+
<i>Proteus rettgeri</i>	—	—	—	++	—	—	—	—

AG = acid and gas,

¹ + = slight urease production in 24 hours, increasing on further incubation

++ = slight urease production in 6 hours or less, reaction complete in 24 hours

Urease production demonstrated on Christensen's urea agar

Other biochemical reactions of Group IV paracolon bacilli are shown in Table I. These organisms have an IMViC formula of — + + +, and weak and delayed utilization of sodium citrate is characteristic of the group. Lactose is fermented slowly (2–3 weeks) by two strains, while no definite fermentation by the other four strains was observed after two months incubation. Other carbohydrates are fermented in 24 hours. These organisms are weakly aerogenic, some strains being completely anaerogenic on first isolation but producing small amounts

of gas in subcultures. In all cases dextrose is fermented with acid and gas, but the production of gas in the fermentation of other carbohydrates is irregular and inconsistent. Gas formation in lactose Durham tubes has not been observed, while in the fermentation of sucrose, xylose and mannitol gas may or may not be produced.

No description of the organisms included in Group IV could be found in the literature. Although the number of strains is too small to be significant in respect to probable pathogenicity, it should be noted that all were isolated from patients with diarrhea and that efforts to find similar organisms in other clinical material have been unsuccessful to date.

DISCUSSION

The differentiation of paracolon bacilli here suggested presents nothing radically new or different, but follows the pattern already established by the reports of other investigators. It follows the suggestion of Stuart and his associates (Stuart, Mickle and Borman, 1940, Stuart and Rustigian, 1943, Stuart, Wheeler and McGann, 1946) that the paracolon group includes the aberrant coliform bacilli and also other Gram negative bacilli which appear to be intermediate between the coliform group and various non-coliform genera such as *Salmonella*, *Proteus* and *Shigella*. Under the system of differentiation just described, the Group I paracolon bacilli, which behave as do the coliform bacilli on CI and SS agar, appear to be aberrant coliforms, while Groups II, III and IV appear to be intermediate forms between coliform and non-coliform organisms.

In our laboratory the determination of growth or inhibition on either the coliform-inhibiting (CI) medium described, or on Difco SS agar, has served as a useful screen test in separating the aberrant coliforms of Group I from the other paracolon bacilli. However, the value of the test, in our opinion, lies not so much in its use in classifying the paracolon bacilli as in differentiating between the aberrant coliform bacilli of Group I and *Salmonella*, *Shigella*, and *Proteus*. Since the paracolon bacilli commonly encountered in various types of clinical material, particularly urine cultures and normal stool specimens, are of Group I whose growth is inhibited on coliform inhibiting media, this test has proved a valuable aid in the rapid identification of paracolon bacilli from human sources.

In the course of the investigation leading to the development of the new coliform-inhibiting medium, all the Gram negative bacilli in our collection were repeatedly tested over a period of ten months. During this time no alteration was noted in the inhibition of the typical coliforms or the Group I paracolon bacilli, or in the growth of stock cultures of Groups II, III and IV paracolon bacilli and the control cultures on C I or S S agar. However, preliminary studies of variants of paracolon bacilli have shown that rough and small colony variants of Groups II and IV paracolon bacilli fail to grow on C I and S S agar, while the smooth forms of these organisms consistently give good growth on these media. Stuart, Wheeler and McGann (1946) have reported similar inhibition in colonial variants of Type 29911 strains (Group III). With these exceptions, the characteristic of growth or inhibition on coliform inhibiting media appears to be a stable characteristic, and one that can be depended upon for the identification of newly isolated strains of paracolon bacilli.

The number of strains of paracolon bacilli included in our series is too few to draw any definite conclusions as to the relative pathogenicity of the four groups here described. However, in view of the fact that evidence for the etiological role of some strains of paracolon bacilli in gastro-enteritis is accumulating in the literature (Barnes and Cherry, 1946, Stuart and Rustigian, 1943, Stuart, Wheeler and McGann, 1946, Galton, Hess and Collins, 1947, Christensen, 1947), the source of our strains is interesting and possibly significant. Fifteen strains of paracolon bacilli received from Dr. Parr were isolated from normal feces, and all were inhibited on S S and C I agar and so classified as Group I. The remaining twenty-two strains of Group I paracolon bacilli were isolated from a variety of clinical material, primarily urine cultures, stool specimens and autopsy material. Their occurrence is, therefore, the same as that of the typical coliforms and such as would be expected of aberrant coliform bacilli. The sixteen strains comprising Group II-A, Group III and Group IV were all isolated from patients with diarrhea or gastro-enteritis. No strains of these groups have been isolated from normal stool specimens or from other clinical material. Since Groups II, III and IV paracolon bacilli are not inhibited on S S agar or C I agar and are, therefore, differentiated from the typical and aberrant coliform bacilli of the normal intestinal tract, and since these organisms appear to be intermediate forms between

coliform bacilli and *Salmonella*, *Proteus* and *Shigella*, their etiological significance in intestinal disease as suggested by their occurrence in pathologic stool specimens does not seem improbable

SUMMARY

1 On a simplified desoxycholate-citrate-thiosulfate medium (coliform-inhibiting or C I agar) and on Difco S S agar, the growth of typical coliform bacilli is inhibited. On either of these media, using a properly diluted culture as an inoculum, the growth of a large number of paracolon bacilli is also inhibited.

2 Correlating growth or inhibition on C I or S S agar with biochemical characteristics, the paracolon bacilli in our series fall into four large groups and a number of sub-groups.

Group I—aberrant coliform bacilli, consisting of paracolon *Escherichia*, paracolon *Acrobacter* and paracolon intermediates, growth inhibited on coliform inhibiting media.

Group II—hydrogen-sulfide-producing paracolon bacilli, growth not inhibited on coliform inhibiting media. This group may be divided culturally into three sub-groups, II-A, II-B and II-C.

Group III—anaerogenic paracolon bacilli, growth not inhibited on coliform inhibiting media (Stuart Type 29911).

Group IV—malonate-positive paracolon bacilli, growth not inhibited on coliform inhibiting media.

3 The use of C I and/or S S agar to determine growth or inhibition of an organism suspected of being a paracolon bacillus is recommended, not only for the possible classification of paracolon bacilli but also for the differentiation of the more commonly occurring Group I organisms from *Salmonella*, *Shigella*, *Pseudomonas* and *Proteus*.

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IRRADIATION OF LYMPHOID TISSUE IN DISEASES OF THE UPPER RESPIRATORY TRACT

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PHYSIOLOGICAL FUNCTIONS OF THE UPPER RESPIRATORY TRACT

The chief function of the upper respiratory tract is the modification of aspirated environmental factors. As in all complex mechanisms any one of a number of small defects may cause more or less serious limitations of function. From the medical point of view our chief interest is in the maintenance of normal function and the recognition and correction of minor defects before major difficulties arise.

Ordinarily, the air about us is below body temperature. It is always lower in humidity than the air in our lungs which is saturated with moisture. Frequently it contains a large quantity of dust and occasionally large foreign particles. Almost any bacterium or virus may be air borne and commonly travels on particles of dust. In ordinary respiration, 500 cc of air passes through the nose on its way to the lungs in about two seconds. During this rapid passage gross particles of dust are removed at the nostrils and smaller particles become imbedded in the nasal mucus (1). The larynx is an additional protection against the passage of large foreign particles into the lungs. The inspired air is warmed to approximately body temperature and its water content greatly increased by its passage over the nasal, pharyngeal and tracheal mucous membrane (2).

Division of the nasal passages into two major parts by the septum and numerous smaller passages by the turbinates serves to provide a remarkably large mucous membrane area within the nose. In the normal nose the passage for air is at no point much wider than four or five millimeters, if both the turbinates and septum were removed, the inside of the nose would consist of a single cavity about 20 millimeters in width. This wide surface area provides a highly efficient humidifying and thermal control apparatus and an excellent opportunity for the removal of foreign material. The several smaller air streams into

which the main column of air is divided allows its passage with less turbulence

The nasal mucous membrane, except at the vestibule and in the olfactory area, is composed of ciliated columnar epithelium. It is highly vascular and rich in mucous glands. Its thickness and vascularity can alter markedly with normal environmental fluctuations and with changes due to disease. The most important characteristics of this mucous membrane are its large content of glands which provide a continual production of thin, tenacious mucus, and its ciliated epithelium (3). The ciliary movements throughout the nose are continually towards the nasopharynx (4-6). It has been calculated that ciliary action in the mucous membrane of the frog is so strong that in one minute one ciliated cell does work sufficient to lift its own weight 4.25 meters. This ciliary activity is extremely persistent in the face of widely varying conditions. A drop in temperature to 5° centigrade suspends ciliary activity only temporarily. Drying and excessive heat will incapacitate the cilia for a longer period. Ciliary activity remains normal as long as the nasal pH remains within the range 5.5 to 6.5 and it will often persist over a much wider range of pH change. During nasal disease, the pH of the nose becomes more alkaline, rest and sleep tend to restore the pH closer to the normal acidity. Ciliary movement within the sinuses moves any foreign material or mucus towards and through the sinus orifices where it joins the stream of mucus that is continually moving towards and into the nasopharynx. According to Hilding (4), mucus moves at a rate of four to six millimeters a minute, and small particles of foreign material have been observed to travel from the anterior end of the middle turbinate to the region of the eustachian tube orifice in four to ten minutes.

One of the commonest potential sources of ciliary trauma is nasal medication. Improper nasal medication may completely cripple the defenses of the nose in a matter of minutes. The use of an oily preparation overburdens and clogs ciliary action and at times causes complete cessation of ciliary motion. Two per cent sodium chloride solution or plain tap water is harmful to the nasal mucous membrane. Solutions of sodium salts of sulfonamides may permanently impair the function of cilia. Silver salt preparations may produce severe caustic

reactions on the mucous membrane and in addition may result in the deposit of silver in the skin, argyria (7-9)

There are several drugs which are harmless when administered in normal saline. Among these are neo-synephrine, ephedrine and penicillin. These drugs are most useful for nasal medication in the following concentrations—neo-synephrine $\frac{1}{4}\%$, ephedrine 1%, penicillin 1000 units per cc. Although the benzedrine inhaler has disadvantages in being a central nervous system stimulant and in having a very short action, it is harmless to the nasal mucous membrane and useful in some instances. There are, of course, other drugs that are beneficial in selected cases, but many of the nose drops in wide use are both harmful and useless. In any nasal medication the first question should be its possible toxic effects on the cilia and the mucous membrane of the nose.

Although a large surface area of mucous membrane and small airways are essential for optimum physiological function, these factors are peculiarly susceptible to serious interference from minor changes. Diffuse moderate inflammation or thickening of the mucous membrane, or minor anatomical abnormalities, may obstruct the passage of air through the nose or block the orifices of the paranasal sinuses. A septal deviation of two millimeters strategically located may upset the normal functioning of the nose. Diffuse thickening of the mucous membrane either from mechanical irritation, allergy, or infection may produce the same undesirable result. Abnormally functioning nasal passages may cause trouble in one of three ways: interference with the sense of smell, interference with the passage of air into the lungs, and by serving as a seat of infection. These disorders may be facilitated by structural defects, damaged mucous membrane, impairment of the ciliary function and the secretion of mucus, or by infection. It is important to note that damage to any part of the nasal passages may result in impairment of all other parts. Thus cessation of ciliary movement will result in a change in the character of the mucus, often followed by infection and thickening of the mucous membrane and interference with the airway. As long as there is normal ciliary function and normal production of mucus and no major mechanical interference with the airway, the whole system is safe with one exception. If the nasopharynx becomes the seat of a fulminating infection, the ears,

nasal mucous membrane and accessory nasal sinuses may become involved

The real vulnerable point in the upper respiratory tract is the nasopharynx. This is true for two reasons: all air-borne organisms gaining access to the interior of the body pass through the nasopharynx and over the adenoids.

Since the discovery of the "lacteals" by Asellius in 1622, the function of the lymphatic system has been a subject of much conjecture. In 1860, Virchow first postulated the "barrier theory." This took into consideration the strategic location of lymphoid tissue throughout the body and the fact that it is almost impossible for foreign material to pass through lymph glands. In 1898, Pfeiffer and Marx demonstrated the increase in antibodies in lymphoid tissues and bone marrow after injection of heat-killed cholera bacilli into rabbits and guinea pigs (10, 11). In 1935, McMaster and Hudack (12) demonstrated the formation of agglutinins within lymph nodes. In 1939, Rich, Lewis and Wintrobe (13), in connection with studies of the body's reaction to foreign protein, stated that "it is tempting to suspect that they (lymphoid cells) may be concerned in the same way in the process of antibody formation." In the same year Sabin (14) said that "one may stimulate the phagocytic cells either of the liver and spleen or of the tissues and lymph nodes to produce antibodies."

Thus, over a period of several decades, there had been a gradually increasing conviction among those working in the field that the entire lymphoid system might be involved in the process of immunity (15, 16). No concrete proof of the manner and extent to which this was true existed until the work of Dougherty, Chase and White in recent years (17-21).

In 1944, these workers demonstrated in studies of extracts of tissues a concentration of antibodies in lymphocytes. In the following years a brilliant series of investigations by these workers have thrown a great deal of light on the entire subject. Working with mice and rabbits and on the basis of certain known facts about the relationship between the adrenals and lymphoid tissue, they were able to demonstrate the release of antibodies from lymphocytes. It was known that lymphoid hyperplasia occurs in Addison's disease and that adrenalectomy causes lymphoid hyperplasia in animals. Also, that adrenotrophic hormone

injected into a normal animal produces dissolution not only of the thymus but of lymphocytes in the circulating blood and lymphoid structures in general. They made extensive studies of the events which follow injection of adrenal cortical hormone or adrenotrophic hormone and came to the following conclusions. Following such injections in normal animals there occurs almost immediately a disintegration of lymphocytes, a washing out of these cells from lymphoid tissues, and extensive changes in the fixed reticular cells. Adrenotrophic hormone will produce none of these changes in the adrenalectomized animal. They were able to conclude that lymphoid tissue contributes to normal defense by

- 1 Release of globulin from lymphocytes, which aids in the maintenance of blood volume

- 2 Release of antibody globulin in the immunized animal

- 3 Increased production of macrophages in lymphoid structures

These workers also were able to demonstrate that the enhancement of antibody titer which follows injection of a variety of non-specific substances other than the original antigen is tied in with this same type of lymphoid tissue activity.

In 1945, Leathart (22) brought up the question of how large a role the tonsils and adenoids may play in the development of immunity, particularly in childhood, and the possible effect on this development of removal of tonsils and adenoids at an early age. It seems reasonable to assume in the face of present day knowledge that the lymphoid tissue in the upper respiratory tract may play an important role in this respect. At first glance, one would believe that the large quantity of lymphoid tissue remaining in the mucous membrane of the nasopharynx, pharynx and at the base of the tongue after the most complete tonsillectomy and adenoidectomy would suffice for these purposes. Yet, on more careful consideration, one can conceive the possibility that no matter how much lymphoid tissue remains most of it lacks the strategic location of the original tonsils and adenoids in regard to its exposure to food and air. Certainly this possibility must be carefully weighed when one is considering the removal of tonsils and adenoids in the young child simply because of recurrent upper respiratory infections. It is possible that their removal may seriously impair the child's ability to acquire immunity (23).

The tonsils and adenoids are susceptible to infection for two reasons

1 Their surface is pitted with crypts which, in the presence of marked hypertrophy, may be deep and intricate. The epithelium lining these crypts always is either partially or totally destroyed by severe acute or oft-repeated infections and replaced with granulation tissue. The resulting scar tissue formation and the secondary keratosis, or horny overgrowth of epithelium around the orifice, interferes with drainage or completely seals off the crypt. In the tonsils this is an especially frequent occurrence and necessitates surgical removal, which must be complete and include all of the crypt system. In the adenoids and hyperplastic lymphoid nodules on the lateral and posterior pharyngeal walls and at the base of the tongue, the crypts are not so deep or branching as in the tonsils, and surgical removal of the larger surface masses, supplemented with irradiation, enables us to flatten out or completely remove the crypts without removing the underlying mucous membrane.

2 Their location exposes them to bacteria entering the mouth in food or drink and to air-borne viruses and bacteria. In the days before milk was pasteurized and before State Health Departments inspected dairy herds and killed all tuberculous cows, it was common to find tubercles in the crypts of tonsils and metastatic tuberculous glands in the neck. There is no such simple way to prevent air-borne organisms, and it is for this reason that the nasopharynx is the starting point for most virus and pyogenic upper air-passage infections and their complications.

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PATHOLOGY OF UPPER RESPIRATORY INFECTIONS

In any discussion of the treatment of hypertrophied or infected lymphoid tissue in the upper air passages, especially in children, it is of interest to review the experimental and clinical observations of the past 25 years concerning

- 1 The etiology of the common cold
- 2 The interaction of virus and pyogenic bacteria in producing ear and sinus complications
- 3 The functions of lymphoid tissue
- 4 The known causes of hyperplasia of lymphoid tissue

Removal of tonsils and adenoids is probably the most common of all surgical procedures, and the number of patients operated on every year would, no doubt, be greatly increased if hospital beds were available to take care of the thousands of children for whom this operation is advised. It is perhaps fortunate that facilities are not available to carry out on all children the recommendation of family physicians, pediatricians, otolaryngologists and school examiners that the tonsils and adenoids be removed. It is not necessary to discuss here the indications and contra-indications for tonsillectomy and adenoidectomy, other than to say that the decision always should rest on as rational a basis for this operation as it does for an appendectomy or other surgical procedures. The end result of a tonsillectomy and adenoidectomy is good in selected cases, but in many children the benefits of the operation, even when it is thoroughly and carefully performed, are only temporary and, a few months or a year later, new symptoms begin to manifest themselves, such as impaired hearing or sinus infections. The explanation lies in the fact that lymphoid tissue is an integral part of the mucous membrane of the posterior and lateral walls of the pharynx and nasopharynx and that all of it cannot be removed. At operation only the large central mass of adenoids and smaller nodules in the fossa of Rosenmüller are taken out. Much lymphoid tissue is left behind, and as a result it not only begins to hypertrophy with the next cold but this regenerated lymphoid tissue contains crypts and crevices, similar to those in the original adenoid mass, that make ideal lodging places for virus and bacteria.

The common cold is one of the most prevalent of human diseases and

is directly or indirectly responsible for many of the conditions the otolaryngologist is called upon to treat. Epidemiological studies of the incidence of this disease show that practically every man, woman and child in the United States has, on the average, about two and one-half colds a year. Susceptibility is greatest in childhood, but continues high even to extreme old age. One of the most important portals of entry of infectious agents into the human body is the respiratory tract. In spite of such protective mechanisms as tortuous channels, an abundance of sticky mucus, ciliated epithelium and aggregations of lymphoid tissue, foreign matter, bacteria and viruses floating in the air are able to lodge in certain parts of the respiratory tract and there to set up irritative or infectious processes.

Search for the etiological agent of the common cold has extended over a period of many years. For a long time the cause was thought to be one or more of the pyogenic bacteria. This idea was dispelled by the observations that healthy individuals harbor in their nasopharynx potentially pathogenic organisms, such as pneumococcus, streptococcus, staphylococcus and *H. influenzae*. Usually, the first appearance of these organisms in the nasopharynx of infants is not associated with an inflammatory reaction of any kind (1). Furthermore, day by day cultures from the upper air passages of large groups of college students show that in the early days of a cold no new organisms appear in the throat flora.

In an isolated community in the far north (4), the arrival of a ship with a single individual with an acute upper respiratory infection has initiated an outbreak of the common cold, which spreads rapidly among the natives, often reaching a morbidity rate of approximately 40% in a few weeks. After the port was closed, because of ice, infection quickly died out and, in spite of extreme exposure and chilling during the winter months, there was no more acute upper respiratory disease until the following summer when communication with the outside world was again established. In this community, no significant change in the bacterial flora of the upper respiratory tract could be demonstrated during the winter when the common cold was almost unknown, and during the spring and summer when colds were most prevalent.

It was shown by Kruse, (2) in 1914, that the common cold could be communicated to experimental subjects by instilling into the nostrils

bacteria-free filtrates of nasal washings from individuals suffering from acute colds. This theory that the common cold is due to virus infection was received with scepticism, until confirmed and conclusively proven to be correct about twelve years later by Dochez and his associates (3). With a long series of carefully controlled experiments on chimpanzees and human volunteers, these observers showed that the virus initiates infection in the upper respiratory tract and produces its own type of disease. Furthermore, it seems to make local conditions in the nasopharynx favorable for secondary pyogenic infection of the surrounding mucous membrane which sometimes extends to the tonsils, sinuses, ears or lungs. In addition, repeated infections with the virus seem to facilitate the distribution of pyogenic bacteria throughout the community. The number of carriers increases and the soil is thus prepared for outbreaks of respiratory disease in the population at large.

Records of the incidence of the common cold show well-defined peaks in the early fall, mid-winter and late spring. The number of infections sufficiently severe to cause absence from work is much larger during the mid-winter and spring outbreak of colds than during the October outbreak. Kneeland and Dawes kept the infant population of a foundling home under observation for several years, studying the character of the respiratory infection in each child, its relationship to the common pathogenic organisms of the upper respiratory tract, and the changes in sensitivity of the skin reactions to products of representative strains of respiratory pathogens. In the early fall, the number of individuals giving significantly positive skin reactions to the various bacterial products was approximately 15%. As the winter progressed, the positive skin reactions became more frequent, and the intensity of the reactions increased, until in the late spring 85% of the group gave significantly positive skin reactions to the pneumococcus, streptococcus and H influenzae. The incidence of pathogenic organisms found by culture varied from time to time. During one year, the carrier rate for H influenzae was high. During the preceding year the carrier rate of pneumococcus rose to the exceedingly high figure of 95%. Throughout the period of widespread distribution of pathogenic organisms, acute upper respiratory infection was usually severe and there was a marked increase in the number of diagnoses of grippe, influenza, pneumonia and their complications, and a corresponding decrease in the

number of diagnoses of common cold. On the other hand, when the distribution of pathogenic organisms was relatively limited, as shown by daily cultures, the great majority of acute upper respiratory infections received the diagnosis of common cold. The conclusion seems justified that certain epidemics of the severe forms of respiratory infection result from the coincidence of an outbreak of the common cold with a period of widespread distribution of well-recognized pathogenic organisms of the respiratory tract.

The nasopharynx is the most important portal of entry of infectious agents in the upper air passages. Pathogenic organisms gaining entrance to the body by way of the respiratory tract fall into two large groups, one of these causes varying degrees of inflammatory reaction in the respiratory organs in the early stages of the disease, to be followed later by evidence of disease in organs distant and unrelated to the respiratory apparatus. Types of such diseases are measles, cerebrospinal meningitis, rheumatic fever, hemorrhagic nephritis and others. The second large group may be called the true respiratory diseases in which the inflammatory process is more or less confined to the respiratory tract itself.

The common cold virus can be grown by inoculating tissue cultures or living chick embryos. The cultures are incubated under anaerobic conditions at 37° Centigrade, and transfers are made to fresh tissue medium every three or four days. The virus will not grow under aerobic conditions. The different generations of virus grown in this way have been inoculated into human volunteers from time to time and have produced typical colds in over 50% of the individuals inoculated. The common cold virus, in common with all other viruses, grows only in living tissue, it differs from bacteria in that it will not grow on dead tissue. The virus must enter a living cell before it can multiply and cause clinical symptoms. The chimpanzee is the only animal known to be susceptible to the common cold virus. Symptoms usually manifest themselves from 24 to 48 hours after intranasal inoculation with the virus, both in chimpanzees and human volunteers. The first subjective symptom complained of by the volunteers is a "hot spot" in the nasopharynx, nasal obstruction, sneezing and abundant mucous discharge. Virus infection never causes a purulent discharge. Then follow hyperaemia of the pharynx mucosa and swelling of lymphoid

tissue Secondary bacterial invasion and purulent discharge usually come in the later stages of the cold The virus, together with the hyperaemia, edema and increased secretion of thick mucus, seems to promote and further a pyogenic infection of the ears, sinuses and other parts of the respiratory tract All evidence suggests that the bacteria of the upper respiratory tract are of themselves powerless to initiate infection (4) (5)

We do not know the mechanism by which the virus enters a cell, but we do know that the virus must be in contact with the cell for at least 30 minutes before it gains entrance or is able to produce symptoms In what part of the upper respiratory tract does the virus find a lodging place for a sufficient length of time to allow it to enter cells and begin to multiply? Why are some children and some adults more susceptible to colds than others? The explanation must be largely mechanical Certainly it is not due to an acquired or induced immunity to the common cold The primary object of all investigators of the common cold has been to find a vaccine, drug or some measure that will prevent or protect against colds, and so far all efforts in this direction have been a failure Many large studies, carefully conducted with adequate controls, have shown no real difference in the incidence of colds following the use of stock bacterial vaccine, autogenous bacterial vaccine, or subcutaneous injections, at weekly intervals, of the common cold virus grown in tissue culture and from time to time passed through apes or volunteers (5)

Antibiotics and sulfonamides control the acute bacterial complications of the common cold but have no effect on the virus and will not prevent recurrences Based on the findings of early investigators that chronic respiratory disease of rats on a diet deficient in Vitamin A could readily be cured by feeding Vitamin A, large amounts of Vitamins A and later D and B have been given to children, but it was found that in general they fared no better in regard to the occurrence of respiratory disease than do those on an ordinary diet In addition to its antirachitic properties, ultraviolet light has been proposed as an aid to general health, and this agent has also been tried as a prophylactic measure against colds Well controlled studies on this subject on a large group of college students indicate that regular exposure to ultraviolet rays does not diminish the incidence of colds Experiments on

college students have also shown that neither hygienic sleeping conditions, the kind of clothing worn, or an abundance of out-door exercise have any appreciable effect in lessening susceptibility to the common cold or increasing the resistance to respiratory infections (6)

Following surgical removal of tonsils and adenoids in children, however, otolaryngologists and pediatricians have repeatedly observed a marked diminution and, in some cases for the period of a year, an almost complete absence of colds. This fact also suggests a mechanical factor in the cause of upper respiratory infections. Reports in the literature indicate that there is no statistically significant decrease in the incidence, severity, and type of upper respiratory infections after removal of tonsils and adenoids. Such studies, however, have failed to take into account the tendency for lymphoid tissue to regenerate. The mucosa of the posterior and lateral walls of the pharynx, and especially that of the nasopharynx, is rich in lymphoid tissue. Indeed lymphoid tissue is an integral part of the mucous membrane in these regions, and for this reason it is impossible to remove all of it surgically. In a group of 1365 school children examined with a nasopharyngoscope, 755 had had their tonsils and adenoids removed at various hospitals in Baltimore. As a rule, the tonsils had been cleanly removed, and in many the oropharynx looked normal, but in more than 75% adenoid tissue had recurred in the nasopharynx (7). We have no data regarding the incidence of colds before and after operation in this group, but mention these observations in order to emphasize that recurrence in the nasopharynx after operation is not only frequent, but that the regenerated lymphoid tissue usually contains crypts and folds similar to those seen in normal adenoids. As will be shown later, these crypts and folds make ideal lodging places for air borne viruses and bacteria and are probably of prime importance in the epidemiology of upper respiratory infection.

Lymphoid tissue is one of the three important protective mechanisms in the upper air passages. The other two are 1) the layer of mucus that is constantly being secreted by the nasal mucosa, and 2) ciliated epithelium that keeps this layer of mucus moving backward into the nasopharynx and pharynx, where it is joined by saliva from the mouth, finally flowing into the esophagus. Lymphoid tissue is most abundant in the midline of the nasopharynx and in the fossa of Rosenmuller,

but after surgical removal of adenoids in children nodules of this tissue often appear in and around the pharyngeal orifice of the eustachian tubes, in the choanae near the posterior end of the middle turbinates, and on the posterior and lateral surfaces of the vomer. These facts concerning the distribution of lymphoid tissue in the nasopharynx and posterior part of the nose are important for a clear understanding of recurring colds, of certain types of impaired hearing, attacks of suppurative otitis media and mastoiditis, ethmoiditis and chronic post-nasal discharge.

We speak loosely of acute tonsillitis and nasopharyngitis as an infection of lymphoid tissue, but histologic examination of tonsils and adenoids that have been removed soon after an acute infection rarely shows an abscess or even any large number of polymorphonuclear leucocytes or bacteria in the lymphoid tissue itself. The most prominent changes are seen in the crypts, where the lining epithelium is partially or completely destroyed (8). Lymphoid tissue itself is very resistant to infection by the common pyogenic bacteria. It is often invaded and destroyed by the tubercle bacillus, the typhoid bacillus and a few other organisms. The chief functions of lymphoid tissue are to protect against pyogenic invasion and to aid in the manufacture of antibodies. This is probably the reason why there is so much lymphoid tissue in the mucous membrane of the nasopharynx and pharynx and in the intestines, the two areas most exposed to bacterial invasion.

The life cycle of the lymphocyte is short, and Dougherty and White have shown that by dissolution they contribute proteid (serum gamma globulin) to the blood and thus help to maintain blood volume. This normal dissolution process is under adrenal cortical control. In adrenalectomized animals, lymphoid tissue throughout the body hypertrophies because the normal dissolution process is slowed or stopped. On the other hand, injection of adrenal cortical hormone in a normal animal accelerates disintegration of lymphoid tissue, and within an hour degenerative changes are seen in germinal center cells, and lymphocytes rapidly disappear from the blood. These changes prevent replacement of lymphocytes as they are used up, and lymphoid tissue throughout the body atrophies. Alterations in lymphoid tissue do not occur following similar injections in adrenalectomized animals. It is probable that infection stimulates the formation of adrenal cortical

hormone, which in turn greatly increases the disintegration of lymphocytes, thus releasing antibody globulin (9) These facts integrate the role of lymphocytes and adrenal cortex in the normal and pathological physiology of the organism, and clearly indicate that lymphoid tissue in the upper air passages, as elsewhere in the body, has a function When hypertrophied or infected, therapy should cure the patient and prevent recurrence of symptoms, but should be as conservative as possible so far as removal or destruction of normal lymphoid tissue is concerned

The common cold virus usually is air borne, but before it can produce symptoms it must find a lodging place in the upper air passages and remain undisturbed by the flow of mucus and in contact with a cell for at least 30 minutes Experimental studies indicate that this length of time or more, is necessary for the virus to enter living cells and begin to multiply and produce symptoms Clinical observations suggest that the virus lodges in crypts and crevices in adenoids and in the hyperplastic nodules of lymphoid tissue that almost invariably appear in the nasopharynx after surgical removal of tonsils and adenoids We have repeatedly seen children operated on for recurring upper respiratory infections who were entirely free of colds for six months or a year after the operation When these patients again begin to have frequent colds, examination with a nasopharyngoscope usually shows nodules of lymphoid tissue in the nasopharynx These nodules contain crypts, and evidence that these crypts are serving as a portal of entry for the virus is furnished by the marked decrease in the number and severity of colds that follows a course of irradiation treatments of the nasopharynx Irradiation (10) acts on germinal center cells (Fig 2), stops mitosis and the formation of new lymphocytes to replace those that are constantly being lost by the normal dissolution process—thus causing the hyperplastic nodules of lymphoid tissue gradually to shrink and disappear, obliterating the crypts and leaving in their place smooth, normal-looking mucous membrane and no favorable places for the virus to lodge and grow in the nasopharynx

Many careful studies of large groups of individuals have shown that streptococcus, pneumococcus or H influenzae may be present in the nasopharynx for long periods without signs or symptoms of infection These organisms grow in the crypts and crevices of lymphoid tissue in

the nasopharynx as they would in a test tube. An acute upper respiratory infection, due to the common cold virus, activates these bacteria and possibly increases their virulence. This is the probable explanation for the ear, sinus and bronchial complications of a cold. These bacterial complications of a cold can be cured in the acute stage by the administration of sulfonamides and antibiotics. It is possible with these drugs to clear temporarily the upper air passages of gram positive bacteria. If the drug treatment is prolonged and intensive, the gram positive pathogens disappear but are replaced with gram negative organisms, the most common being the colon, proteus and Friedlander bacilli. These in turn are activated by recurring virus infections, the ear, sinus and bronchial complications recur but this time with gram negative organisms. Streptomycin and penicillin, either separately or in combination, may be essential but certainly are only a part of the treatment. It is impossible to free permanently the upper air passages of bacteria, but if these bacteria are relatively harmless until activated by virus infections, and the principal area in which the virus lodges and begins to grow is the nasopharynx, it is obvious that, in order to lessen or obviate virus infections, we must supplement the drug treatment by removing the crypts and crevices in nasopharyngeal lymphoid tissue with operation and irradiation.

IRRADIATION OF ADENOIDS

It is for these reasons that we urge otolaryngologists to concentrate their attention on the nasopharynx. If symptoms improve following operation or irradiation, we must realize that neither of these measures remove all lymphoid tissue in the nasopharynx. It may regenerate in the fossa of Rosenmuller, on the posterior part of the septum and in the choanae, and symptoms recur. Therefore, the patient should be examined with a nasopharyngoscope at least once a year, preferably during the summer months, and, if necessary, further irradiation given to tide him over the winter months. In this way we feel sure we can prevent in many children impaired hearing due to eustachian tube obstruction or suppurative otitis media, recurring infection of the nasal sinuses, and, in selected cases, lessen the severity and frequency of bronchial asthma attacks (11).

It has long been the custom of the family physician, the pediatrician,

school doctors and otolaryngologists to advise removal of tonsils and adenoids because they are hypertrophied and interfere with speech and nasal breathing, or because the children have repeated upper respiratory infections, with or without some of the pyogenic complications. Of course it is necessary to relieve symptoms and restore the child to health, but we doubt that many of the tonsillectomies now being done are necessary or advisable. This conclusion is based on the results of irradiation of the nasopharynx in the out-patient department of The Johns Hopkins Hospital during the past ten years.

Up to this time, we had used irradiation therapy almost exclusively for the treatment of certain types of impaired hearing. During the depression years, preceding the outbreak of war, the hospital found it increasingly difficult to furnish free beds. It was then we began to treat the nasopharynx with radium for complaints which we previously had thought justified operative removal of tonsils and adenoids. We were forced to treat only the nasopharynx because it is not safe for the doctor to hold a radium applicator in his hand and apply it to the tonsils or to hyperplastic lymphoid tissue in the pharynx. When irradiating the nasopharynx, the patient lies on a couch and the doctor passes the applicator along the floor of the nose until it is in contact with the lymphoid nodules he wishes to remove. He then withdraws to a safe distance. Our previous experiences in treating patients with various types of ear disorders had suggested that the nasopharynx is the primary seat in most upper air passage infections, but we had not expected the beneficial results that soon became apparent in this group of ambulant patients. Acute infection of the accessory nasal sinuses, ears, and tonsils can be cured easily with antibiotics or sulfonamides, together with local measures that promote drainage, but so long as the adenoids with their crypts and crevices remain, conditions are favorable for subsequent pyogenic infections, initiated by the common cold virus, which again may spread to the ears, sinuses or tonsils. As patient after patient with recurrent acute attacks and, in some cases, with chronic infection of the ears and sinuses got well and remained well under this combination type of treatment, the number of patients treated in the radium clinic of the out-patient department was increased. For several years from 50 to 75 patients have been treated each week with results on the average far better than those previously

obtained from operation. To get good results, however, the patients must be carefully selected. Usually the first few infections of the middle ear, sinuses, and tonsils do not leave permanent changes in the lining mucosa or epithelium in the crypts, but after repeated acute infections irreversible changes occur that mechanically interfere with drainage, and surgery becomes a necessary part of the treatment.

Aside from selection of suitable cases, two other factors contribute to the success of this type of treatment. Irradiation in the proper amount and at the proper intervals shrinks lymphoid nodules on the posterior part of the septum, in the choanae near the posterior end of the middle turbinate, and around the orifice of the eustachian tubes, all locations inaccessible to surgical removal. If the dosage is increased, all lymphoid tissue in the nasopharynx can be permanently removed, but excessive dosage always is followed by the appearance of telangiectases in the mucous membrane and dryness and crust formation in the nasopharynx, which are neither necessary nor desirable. A course of five treatments at two week intervals with the smaller dosage, ($8\frac{1}{2}$ minutes on each side) with the 50 milligram 0.3 mm. monel metal radium applicator, or three treatments with the maximum dosage of 12 minutes on each side at two week intervals, has the desired effect and may be repeated with safety once a year if symptoms recur. This leads to the second factor that contributes to success in controlling the incidence of colds and their complications. After every operation or irradiation of the nasopharynx, the patient must be followed and examined at least once a year for several years, and every effort made to keep the upper air passages in good condition, just as the dentist examines his patients at regular intervals in order to keep the teeth in good condition.

Observations on these dispensary patients, on a large group of school children, and on several hundred private patients, confirmed and strengthened our impression that removal of infected adenoid tissue will prevent recurring colds and their complications more effectively than any of the measures cited above. Often irradiation is all that is required, but sometimes the combination of surgical removal, supplemented with antibiotics and irradiation, is necessary to get the best results. The primary object of our study of school children (7) was to determine the relation of hyperplastic lymphoid tissue in and around

the orifice of the eustachian tubes to impaired hearing. All children were tested individually in a sound-proof room with the voice, audiometer, and tuning forks, and then examined with a nasopharyngoscope. In those found to have hearing impairment every effort was made to maintain the nasopharynx in as near normal condition as possible. Others who were similarly examined and found to have hypertrophied lymphoid tissue but no hearing impairment were not irradiated and served as controls. For two years examinations and tests were made at frequent intervals. Then followed a period of four years, during the war, when they were not seen. In 1946 we were able to trace and re-examine 600 of the original 1365 children. This group contained both controls and treated cases. The hearing was found to be improved, with return of threshold acuity for all tones to normal or near normal in somewhat more than half of the treated children, who had had a 25 to 35 decibel loss for all tones. In 1939, when first seen, most of these 600 children gave a history of frequent colds. When questioned in 1946 regarding their susceptibility to colds and sore throats, the terms "rarely", "never", "once a year", "two years since the last one", "mild when I have one now", "they last only a few days", etc. were used far more often by those in the treated group than by those in the control group who had had no treatment.

If nodules of lymphoid tissue are seen in the fossa of Rosenmüller, in the choanae and on the posterior part of the septum, the patient's history almost invariably indicates that upper respiratory infections are frequent, often with ear, sinus or other complications. On the contrary, if no lymphoid tissue is seen in these locations, colds are usually infrequent, mild, and rarely cause loss of time from work or school. Examination with a nasopharyngoscope is necessary in order to visualize these areas. It is not the size but the location and contour of lymphoid tissue that is important. Lymphoid tissue in these critical areas is inaccessible to surgical removal. The presence of a small mass of adenoids in the midline of the nasopharynx has but little effect on the incidence of colds. The important areas are the choanae and the lateral walls of the nasopharynx. Nasopharyngoscopic examination of a patient with a coryza or sinus infection shows a stream of discharge flowing from the choanae into the fossa of Rosenmüller, not into the midline of the nasopharynx (Fig 1). If this is the

normal pathway of the flow of mucus from the nose into the nasopharynx, it is easy to understand how the virus finds a lodging place in the crypts of lymphoid tissue in these areas, why colds recur when

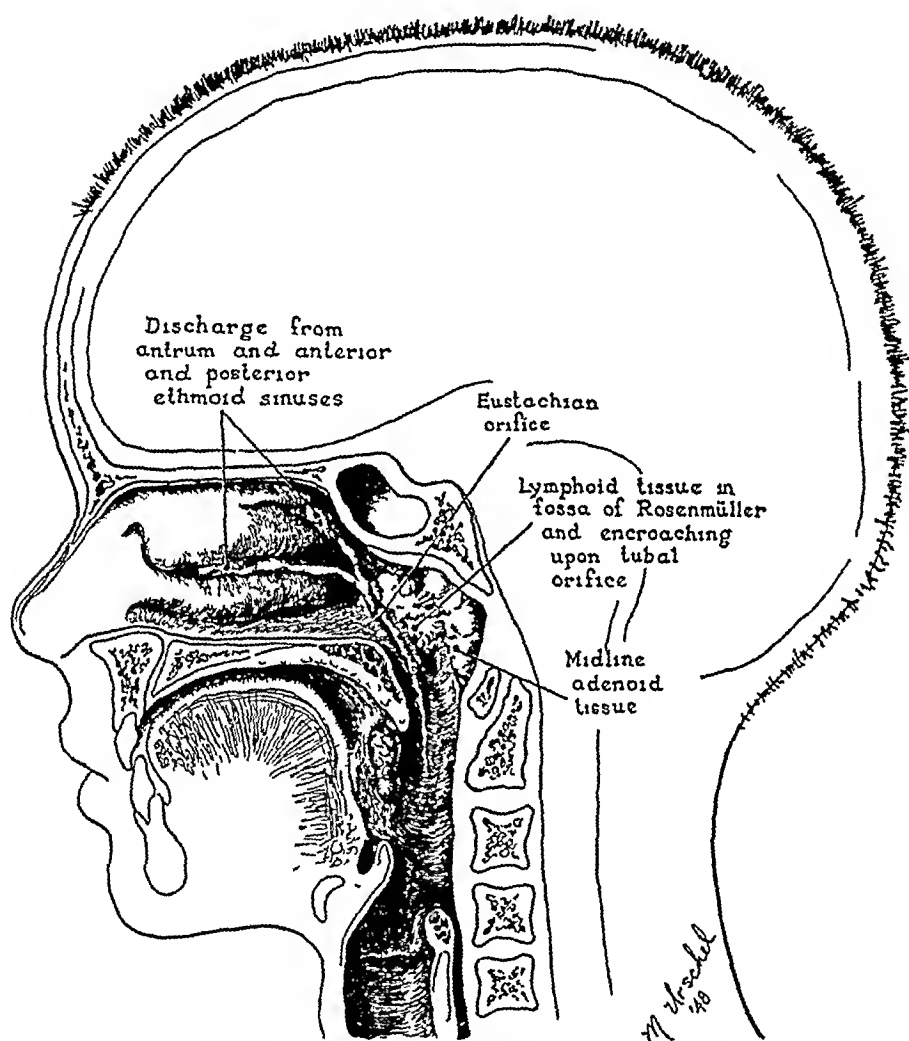


FIG 1 Drawing showing anatomical relationships of the nasopharynx

hyperplastic nodules appear in these areas after removal of tonsils and adenoids, and why colds become less frequent or entirely cease if lymphoid tissue in these areas is kept down with irradiation

The tonsils and adenoids should be removed surgically if the patient has repeated attacks of tonsillitis. Hyperplastic nodules of lymphoid

tissue, which often appear on the posterior and lateral walls of the pharynx after this operation, may also have to be removed surgically if they become chronically infected, especially if the patient has a severe cough and asthmatic bronchitis following every cold. The mere presence of a "granular pharyngitis" however does not always demand operation, cauterization, or other forms of local treatment. Often the symptoms may be greatly improved or entirely cured in these cases by irradiation of the nasopharynx alone. If symptoms recur, a second or third course of irradiation may be given with safety, provided the proper dosage and intervals between courses are observed. Irradiation of the nasopharynx may thus be used to tide a child over a difficult period. Antibiotics are used to cure the acute manifestations, but irradiation is used to remove the underlying cause of upper air passage infections. The combination prevents recurrences and thus aids in obtaining a permanent cure.

The impression we wish to convey in this publication is that irradiation of the nasopharynx with a radium applicator is no panacea, but in selected cases it is an invaluable therapeutic measure. Anyone who has had a series of common cold infections knows that they do not always begin with a nasopharyngitis. Sometimes the virus gains entrance through the nasal mucosa, injures cell membranes, and thus prepares the way for secondary bacterial invasion. There is abundant evidence, however, that the more severe types, with complications, begin in the nasopharynx. The acute symptoms can be controlled with anti-bacterial drugs, but to prevent recurrent infections and the possibility of permanent injury to ear and sinus mucosa, hyperplastic nodules of lymphoid tissue in the fossa of Rosenmüller, in the choanae, on the posterior and lateral surfaces of the vomer, and in and around the orifice of the eustachian tubes must be searched for and irradiated—not excessively, but enough to keep them small and as free as possible from infection.

Before discussing the technique of irradiation with the nasopharyngeal radium applicator it is profitable to review some of the general factors in irradiation therapy (12).

X-rays and gamma rays of radium, rays of ordinary visible light, ultra-violet and infra-red rays, as well as radio waves, are similar in many respects. They all have the same speed, 186,000 miles per

second They have no electrical charge They can be polarized, reflected and diffracted The only difference is their frequency or wave length The shortest wave lengths are obtained from the gamma waves of radium and from extremely high voltage x-ray There is a direct relationship between wave length and energy, the shorter the wave length, the greater the energy, i e , the penetrating power

Beta rays vary greatly as to speed Some closely approach that of light Approximately 93 per cent of the beta ray output of an applicator is absorbed by one centimeter of tissue For this reason beta rays are valuable in treating many superficial conditions on the skin, in the nasopharynx, and the eye A different type of applicator (screen), however, is used in each of these locations The 0.3 mm monel metal radium applicator is designed for the treatment of hyperplastic lymphoid tissue in the nasopharynx, and is not suitable for the treatment of malignant growths in the nasopharynx or nose, or benign lesions of the skin or conjunctiva

When high penetration is desired it is very important to remove by filtration all beta rays Gamma rays from radium have a capacity of penetrating tissues (many pass entirely through the body) and great thicknesses of very dense metals, for example, a small percentage of these rays pass through 12 inches of lead It is for this reason that office personnel should not sit day after day at a desk in close proximity to a radium applicator, even though it is in its lead container Always remember that distance affords the greatest protection for the doctor and his helpers

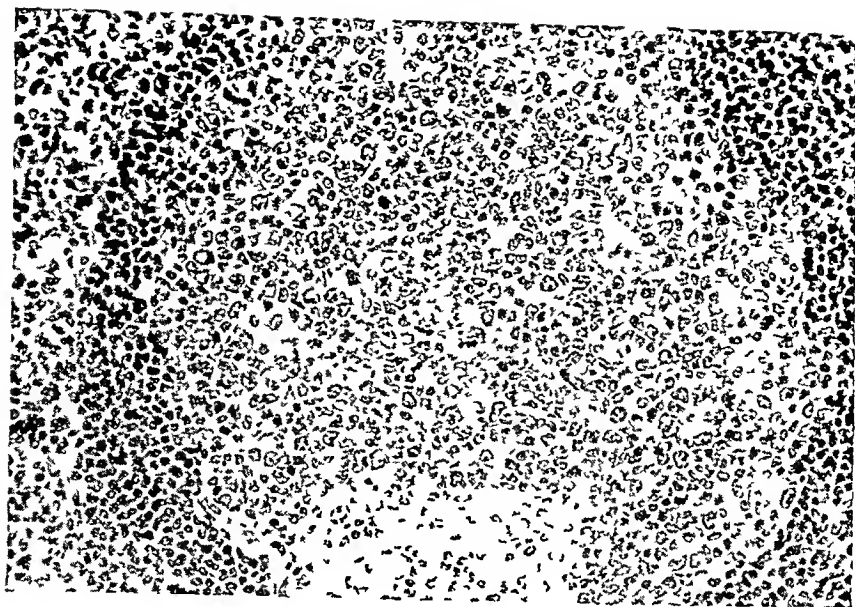
The rays from radon and radium are identical Irradiation may be so weak that no demonstrable changes in cells or tissues can be observed It may be so strong that all cells and tissues it strikes may be completely destroyed Therapeutic irradiation lies between these two extremes What a metal filter does is to remove the longer wave lengths completely It is obvious that this reduces the quantity of rays, but increases the penetration, due to the fact that the average wave length is shorter Some of the rays pass through the body unchanged, these have no biological effect It is practically certain that the rays which are absorbed, whether in whole or in part, produce ions and that these ions cause the biological changes in tissues Both clinical and experimental observations indicate that irradiation is

always an injurious agent and never a direct stimulant. It is true that radiation, which is not lethal and the recovery rapid, may be followed by overactivity in growth, but this is not peculiar to irradiation since it often follows other types of injury. The effects are quite different as the time of exposure is varied. A given amount of irradiation, delivered in a few minutes, may produce a violent reaction on the skin or mucous membrane, whereas if the same amount be given in a period of a month, no reaction will occur. It is certain that it is much better to treat nasopharyngeal lymphoid tissue with three or four doses (just short of the irritating or destructive level), at intervals of two weeks, than by a single massive dose.

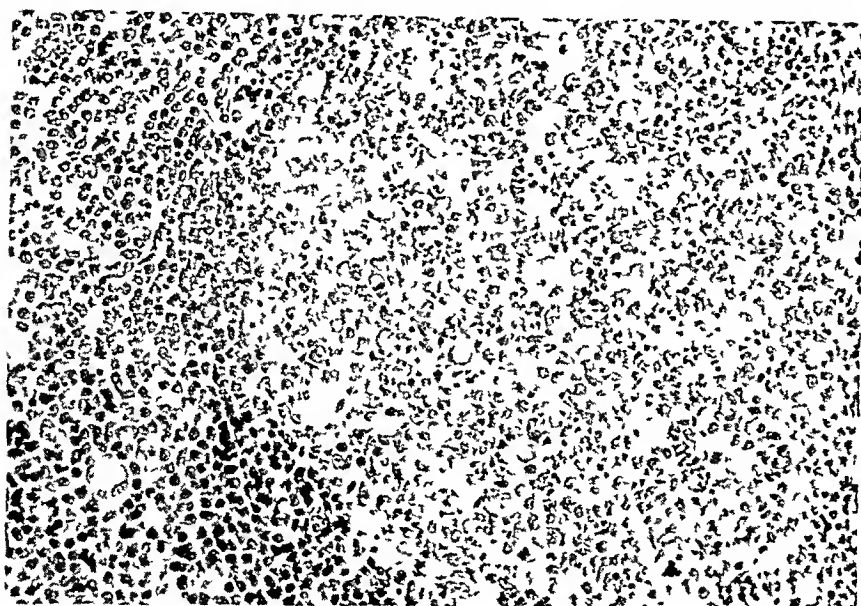
The changes, both gross and microscopic, which take place in cells and tissues after irradiation, are not specific and are observed following various other injuries. Following irradiation, the first changes are seen in the nucleus of the cells (Fig 2). Mitosis and cell division are brought to a halt. The protoplasm of the cell body soon becomes granular. With the ideal irradiation the cell is not destroyed, even cell life is not shortened, but reproduction is prevented. We should strive to get this effect in treating the nasopharynx.

There are great variations in radiosensitivity between different cells and tissues, and indeed individual cells of the same type vary greatly. Moderate irradiation may destroy half or more of the cells of a given tissue, but to destroy every cell may require four or five times the amount of radiation. There is evidence that tissue in the nasopharynx, as elsewhere in the body, plays a role in antibody production and is to be preserved so far as possible. There is also evidence that recurring infections of tonsils and adenoids are due in part to the crypts and crevices in which viruses gain entrance and bacteria lodge and grow. If the contour can be sufficiently changed to flatten out these recesses with operation or irradiation, and at the same time preserve as much lymphoid tissue as possible, infections often become less frequent and less severe and the end result is more ideal than that which follows total removal or destruction of all lymphoid tissue.

The "latent period" is the term applied to the time that elapses after irradiation before definite local reaction changes can be seen. The length of the latent period varies inversely with the intensity of the irradiation. In the skin, slight erythema may occur as late as three



a



b

FIG. 2 Irradiation of the nasopharynx with the dosage used does not cause an immediate change in the gross or microscopic appearance of any of its tissues. Sections of adenoids removed 10 minutes after irradiation (a) cannot be told from sections of untreated adenoids. When removed, 24 hours after irradiation the germinal centers of the lymphoid nodules show a marked degree of fragmentation of the rapidly multiplying cells. (b) All other tissues, including the vascular endothelium, are normal in appearance. Magnification of 450 diameter.

weeks after mild radiation, whereas with very intense radiation it may develop in a few hours. These changes involve both the nucleus and the cell body. Physiologic changes are more difficult to evaluate, but a definite change in metabolism, increased permeability of cell membranes leading to edema, and increased acidity are all common occurrences.

The aim of irradiation treatment always is to cure and not to injure the patient. In ophthalmology it is particularly vital not to injure any of the essential structures of the eye upon which vision depends. In nose and throat work, injury of bone, cartilage and nerve tissues must be avoided, and the dosage is planned so as to reduce to a minimum the possibility of such injuries. In otolaryngology and ophthalmology the beta ray applicators are particularly valuable in treating superficial lesions. For the treatment of deep-seated and extensive (malignant) lesions such as may occur in the orbit, sinuses, nasopharynx, pharynx, tonsils or larynx, x-ray is the best agent. In using x-ray for this purpose accurate cross-firing through small portals is essential. Far too frequently portals 10 centimeters in diameter are used in treating a growth 1 centimeter in diameter. This inevitably means over-irradiation of the surrounding normal tissues.

The technical details of treatment with beta ray applicators are simple. After a short course of instruction any competent ophthalmologist or otolaryngologist can safely and effectively use these applicators on his patients. The course of instruction should include training in the out-patient department in the diagnosis of the various conditions that may be benefitted by this type of treatment, and, under supervision, the actual treatment of patients. Malignant growths, however, should always be treated by the radiologist.

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CLINICAL CONSIDERATIONS

Lymphoid tissue in the pharynx and nasopharynx plays an important part in both the physiology and pathology of the upper air passages. It protects against invasion of the body by pyogenic bacteria, but affords no protection whatsoever against the common cold virus. On the contrary, the crypts of lymphoid tissue, especially in the nasopharynx, make an ideal lodging place and portal of entry for this particular type of virus. The virus damages cell membranes and opens a pathway for bacterial infection, which is directly or indirectly the cause of impaired hearing, sinus infection and a host of other conditions the otolaryngologist is called upon to treat. The physician must not be satisfied merely to restore the hearing, relieve the pain or stop the discharge of acute infections, especially those in the ears and sinuses, but following recovery from the acute symptoms a search must be made for any abnormality in the nasopharynx and upper air passages that might cause a recurrence of the ear or sinus complications with the next cold. In other words, every effort must be made to prevent disability due to impaired hearing, chronic infection of the accessory nasal sinuses and bronchiectasis. The therapeutic measures available include surgical measures and vasoconstrictors to insure adequate drainage, the administration of sulfonamides or antibiotics to combat the acute infection, and surgical operation, irradiation, or both, in an effort to prevent recurrence. The rationale for this lies in the remarkable ability of the mucous membrane in the ears, sinuses and bronchi to return to normal after an acute infection, oft-repeated infections, however, lead to chronic changes in the lining mucous membrane of ears and sinuses that permanently interfere with drainage and necessitate radical surgical operation. Our experience indicates that much good can be accomplished by preventive therapy in children, but if impaired hearing,

due to intermittent blockage of the eustachian tubes, or recurrent sinusitis is not corrected and stopped during childhood, when most of these maladies begin and the changes in the mucous membrane are still reversible, there is little likelihood of curing them in adults

We began in 1924 to use irradiation with a radon applicator for the removal of recurrent lymphoid tissue following adenoidectomy, and particularly for displaced nodules of lymphoid tissue in and around the pharyngeal orifice of the eustachian tubes that often lead to impaired hearing. These treatments proved so effective and so simple and safe that we have used this type of therapy in selected cases with increasing confidence and satisfaction for 24 years. The late Dr Curtis F. Burnam, one of the outstanding authorities on the physics of radium and its clinical uses, was from the beginning the radiological consultant for this work. He has been responsible for all decisions as to dosage, type of filtration, and construction of applicators (1). During this period applicators with various types of filtration have been used—gold, brass, aluminum and monel metal. Determination of the proper dosage for these various types of applicators (i.e. the number of minutes the applicator must remain in contact with the tissue being treated) was of the utmost importance to insure safety and effective results. The dosage we used was not established by mathematical calculations, but by determining the erythema dose of the applicator for buccal mucous membrane. Less than half the erythema dose has always been used in treating nasopharyngeal lymphoid tissue. The clinical results have been satisfactory with this dosage, no complications have arisen, and we are certain that a larger dose is neither necessary nor desirable.

For many years the interval between treatments was one month and most patients were given three treatments of 2 to 2.2 gram minutes through 1 mm. of brass. Only in exceptional cases were more than three treatments given in one year, and then only after consultation with an expert radiologist. During the war years, when it became necessary to speed up the treatments, the interval between each treatment was reduced to three weeks and later to two weeks. These changes were based on observations with the nasopharyngoscope of the reaction following each treatment and how long it persisted. These observations were made on several hundred patients.

As the value of nasopharyngeal irradiation, in the treatment of various conditions that arise from hyperplastic or infected lymphoid tissue, became more and more apparent, inquiries began to come from those engaged in Public Health work among children in rural districts about the possibility of substituting nasopharyngeal irradiation in selected cases for surgical removal of tonsils and adenoids. These inquiries and the demands of the Army Air Forces and Submarine Training School during the early days of the war for some simple way to remove lymphoid nodules in and around the pharyngeal orifice of the eustachian tubes, in an effort to prevent aerotitis, led to the development of the 50 mg radium applicator, screened with 0.3 mm of monel metal (Fig 3). This applicator utilizes to the full the large beta ray out-put that is made possible by the monel metal screen. Only rays that are absorbed by cells are of therapeutic value, those that pass through the



FIG 3 Photograph of the Monel Metal Radium Applicator

body are of no value in treatment. Practically all beta rays are absorbed in the first 10 mm of tissue, and since nasopharyngeal lymphoid tissue is superficial, the 50 mg 0.3 mm monel metal applicator is a satisfactory therapeutic instrument for this area. Gamma rays penetrate to great distances and, like high voltage x-rays, are used to treat deep-seated malignant growths. If platinum or gold replaced monel metal as a screen on the 50 mg radium applicator, the time of each treatment would be greatly prolonged—from 12 minutes on each side of the nasopharynx for the monel applicator to well over an hour on each side of the nasopharynx for the platinum or gold screen. A 1 mm aluminum filter allows the passage of a larger percentage of beta rays than do any of the other metals we have tried, but it is less durable and dependable than monel metal. The applicator becomes practically inert, if even a tiny hole appears in the wall of the radium-containing chamber. The radium-containing chamber must be hermetically sealed, since the first by-product of radium dissolution is radon, and therapeutic beta and gamma rays are formed only from radon. It is evident that if the radon escapes from the radium-con-

taining chamber, no beta and gamma rays will be generated within the applicator and it will be useless

The present applicator has a radioactive length of 15 mm, thus covering the area from a point just anterior to the tubal orifice back to the fossa of Rosenmuller with the applicator in one position

At first, the dosage time for the 0.3 m m monel metal 50 mg radium sulphate applicator was exactly comparable to that of the 50 mc radon applicator with the same type and thickness of screen, but later it was learned that the radium salt itself acted as an additional filter and the dosage time had to be adjusted accordingly At present, the technique of treatment with this applicator is as follows

1 The applicators are stored in a cylindrical lead block with 3 inches (7.5 cm) of lead on all sides



FIG 4 Nasopharyngoscopic photographs showing appearance of normal tubal orifice and tubal orifice partially overgrown with adenoid tissue

2 The nose and nasopharynx have been previously examined with a nasopharyngoscope to determine the suitability of the patient for radiation treatment (Fig 4)

3 The floor of the nose is anesthetized by the passage of a fine cotton applicator moistened with a few drops of 20% cocaine In most instances this is unnecessary as the placing of the applicator occasions little or no discomfort Local anesthesia is chiefly of value in very apprehensive patients or those with severe nasal obstructions

4 With the patient recumbent, one radium applicator is passed into the nasopharynx on each side of the nose until the end lies against (Fig 5) the posterior nasopharyngeal wall in the fossa of Rosenmuller (Fig 6)

5 A timing clock is set for 12 minutes (1 gm 36 sec equivalent) and the physician goes to a distance of about 20 feet

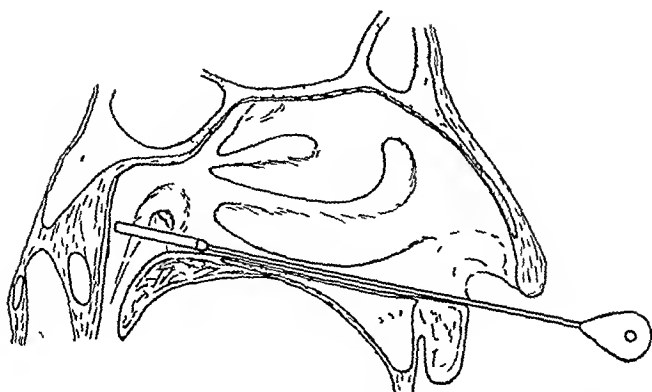


FIG 5 Cross section to show position of radium applicator in nasopharynx

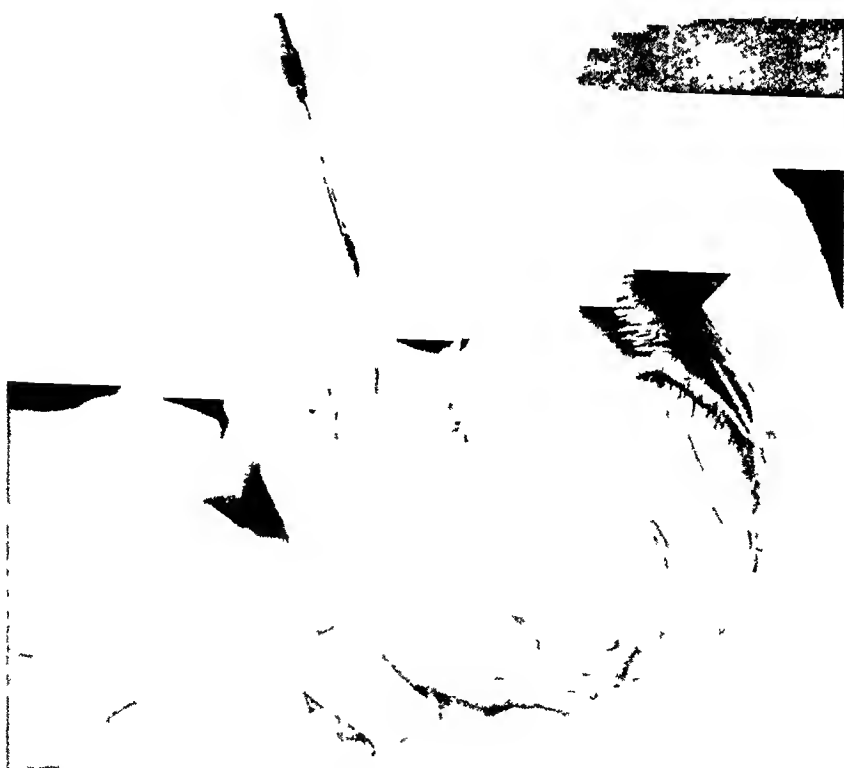


FIG 6 Photograph of child during treatment with applicator in position

6 When the alarm rings, both applicators are removed and quickly cleansed with some automatic cleaning device and immediately replaced in the lead block. The applicators must never be sterilized

with heat Under no condition is the radium-containing capsule ever touched with the fingers

7 Two additional treatments are given at intervals of two weeks

8 Children are followed with periodic examinations at the end of six months, and after that at least once a year The course of treatment may be repeated once or twice at intervals of one year if symptoms recur

This technique is so simple that it can be mastered easily by any physician and is perfectly safe as long as the rules are strictly followed On the other hand, the selection of cases in need of this treatment must be based on careful clinical judgment, after thorough study of the patient's history and examination of the ears, nose and throat, including nasopharyngoscopic examination

It is for this reason that we believe the treatment is best carried out by the otolaryngologist rather than the radiologist

It is impossible to eradicate completely the nasopharyngeal lymphoid tissue by surgery, because lymphoid tissue is an integral part of the mucous membrane of the nasopharynx This lymphoid tissue is exceedingly susceptible to hyperplasia under the stimulus of infection or allergy Irradiation offers a simple non-surgical method of reducing the size of lymphoid nodules

Although our first interest in this form of treatment was aroused by the dramatic improvement in hearing which occurred in some children following treatment, our experiences during the past 24 years have demonstrated its usefulness in selected cases with the following conditions

1 Hearing impairment thought to be partially or wholly on the basis of chronic or recurrent eustachian tube obstruction

2 Chronic otitis media when the concurrent chronic mastoiditis is properly handled

3 Recurrent acute attacks of otitis media

4 Recurrent acute attacks of sinusitis

5 Recurrent acute attacks of pharyngitis or tonsillitis when the infection begins in the nasopharynx

6 Recurrent colds in selected cases

7 Bronchitis secondary to upper respiratory infections

8 Certain patients with bronchial asthma

9 Aerotitis (2-5)

10 In some patients with postnasal discharge, tinnitus, fullness in the middle ear, etc

In all of these conditions, the symptom is the primary consideration. The secondary consideration is whether or not the nasopharyngeal and other examinations confirm the suspicion that the primary focus of infection is in the nasopharyngeal lymphoid tissue. Adenoids are not irradiated unless the patient suffers from symptoms thought to be attributable to them, nor are patients irradiated for symptoms unless there is evidence of the presence of infected adenoid tissue.

If the adenoids are very large they should be removed surgically before irradiation. If there is chronic tonsillitis, sinusitis or mastoiditis these must be treated before or simultaneously with irradiation of the adenoid tissue. Before leaving this section, it would be well to stress the following essential points:

1 In many patients, surgical adenoidectomy alone is not an adequate answer to the problem of infected adenoids.

2 Lymphoid tissue is highly sensitive to irradiation and may be satisfactorily reduced by doses which will not harm the surrounding tissues.

3 This may be accomplished safely and without complications by a method which has been used by us for a quarter of a century.

4 In order to determine those who are in need of therapy, nothing can substitute for careful examination of the nasopharynx with the nasopharyngoscope.

5 Radium therapy of any kind is potentially dangerous, and if one is to avoid all risk to the patient the above rules as to dosage, number of treatments and repetition of treatments must be strictly followed.

RESULTS

It has never been feasible to study an adequate series of controls, because the efficacy of the therapy was apparent in some conditions from the beginning. As a result, any evaluation of the usefulness of irradiation therapy, in alleviating such clinical conditions as those described above, must be based on a comparison with the course of similar patients prior to the introduction of irradiation therapy.

It is well known that adenoid tissue commonly recurs following

adenoidectomy and necessitates repeated surgical removal in some children, also that severe hearing impairment in children seldom regresses spontaneously. Otolaryngologists and pediatricians know that many children continue to have repeated upper respiratory infections or ear infections in spite of surgical removal of tonsils and adenoids. Irradiation therapy changes the clinical picture after operation for most of these children.

One of the authors (Dr Polvogt) recently reviewed 835 of his private patients who received irradiation therapy, often combined with operation, sulfonamides or antibiotics, and who have been followed for at least $2\frac{1}{2}$ years, many for 10 to 15 years. He found that

Of 128 patients treated because of frequent severe colds 40 per cent showed marked improvement

Of 110 patients suffering from postnasal discharge due to nasopharyngeal infection, excluding Thornwald's disease, 25 per cent had complete relief from this symptom and 75 per cent were improved

Of 282 patients suffering from impaired hearing thought to be attributable to obstructed eustachian tubes, 85 per cent had a great and lasting improvement in hearing

Of 122 patients suffering from a variety of complaints thought to be attributable to a focus of infection in the nasopharynx and in whom no other focus could be found, 90 per cent were improved

Of 87 patients suffering from partial nasal obstruction due to adenoid tissue, 90 per cent were completely relieved and the other 10 per cent were improved

(When there is a large amount of adenoid tissue, irradiation always should be preceded by surgery. There is no point in using large doses of irradiation to treat masses of adenoids when surgery will eliminate most of the tissue. Irradiation should be reserved for treatment of small nodules of lymphoid tissue inaccessible to surgery.)

Of 97 patients complaining of tinnitus, only 3 had relief from this symptom, and of 9 patients complaining only of fullness in the ears, 8 of the group were relieved

Although well over a thousand applicators are now in use in this country, to our knowledge not a single physician using them has reported unsatisfactory results.

The reader will obtain a cross section of opinion of various physicians who have used this mode of therapy by referring to the clinical reports listed in the bibliography (6-10).

PREVENTION OF DEAFNESS IN CHILDREN

During the last five years, an extensive trial of irradiation therapy has been made in connection with Public Health Clinics for the Prevention of Deafness in Children (especially the Washington County Clinic at Hagerstown, Maryland) (12-14) The clinic organization is as follows

The clinic is organized as an ear, nose and throat consultation clinic under the auspices of the Department of Public Health One full-day session every two weeks is sufficient to handle the problems of a population of approximately 75,000 people An orientation period prior to

SCREENING OF 3rd AND 6th GRADES

WASHINGTON COUNTY, MARYLAND

SCHOOL YEAR	CHILDREN SCREENED	DEGREE OF HEARING IMPAIRMENT					(%) TOTAL IMPAIRED
		SEVERE	MODERATELY SEVERE	MODERATE	SLIGHT	HIGH TONE ONLY	
1944-1945	1616	25	44	24	57	22	10.5
1945-1946	3118	10	8	32	45	17	3.6
1946-1947	2881	10	19	19	17	7	2.5
TOTAL	7615	45	71	75	119	46	3.4

FIG 7 Data on screening program Washington County, Maryland

the opening of the clinic familiarized the local physicians, school personnel and general population with the aims of the clinic Patients in need of treatment are discovered by testing the hearing once a year of every child in the 3rd and 6th grades (Fig 7), by referrals from private physicians, teachers, and public health nurses, who take into account speech defects and the frequency of ear or upper respiratory infections, as well as direct evidence of impaired hearing At the clinic, complete hearing and ear, nose and throat examinations are made Simple local therapy and irradiation of the nasopharynx are carried out at the clinic When operation, antibiotics or frequent local treatment is necessary in addition to irradiation, patients are referred to local physicians or to the nearest medical teaching center An essential part

of the program is the follow-up by Public Health nurses. All patients are studied over a number of years. Those with irreversible hearing impairments receive adequate help in speech and hearing rehabilitation and, where indicated, properly fitted hearing aids. As yet, this part of our program is still in a developmental stage.

At the Hagerstown Clinic well over a thousand patients have been seen during the past five years. The results listed below are from a statistical study of the records of 400 of these patients who were treated with radium. From our experience, we feel it is safe to say that such clinics are practical and useful. They provide an opportunity to

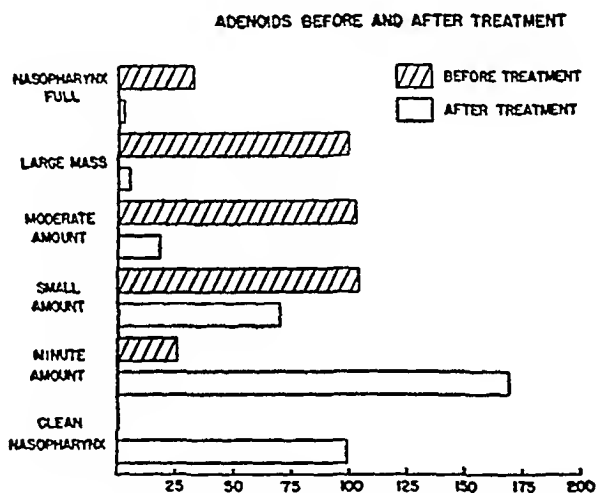


FIG 8 Chart showing given amounts of adenoid tissue before and after treatment

be of major assistance in the most common serious problems of childhood, i.e., ear and upper respiratory infections and hearing impairment. All communities should institute such programs as soon as adequate physicians, Public Health and clinical facilities are available. One of the major advantages of radium therapy is its simplicity. Not only is the patient ambulatory, but only a few minutes are required for the treatment and there is no discomfort or danger for the patient if the physician uses small doses at the proper intervals. Study of 400 patients treated at the Hagerstown Clinic with radium is analyzed in the accompanying charts (Figs 8, 9, 10, 11, 12). Patients were treated with radium because of hearing impairment, recurrent respiratory or

ear infections or bronchial asthma. In most instances the last examination was about a year after completion of therapy, but in a few instances treatment has not yet been completed.

EUSTACHIAN ORIFICES BEFORE AND AFTER TREATMENT

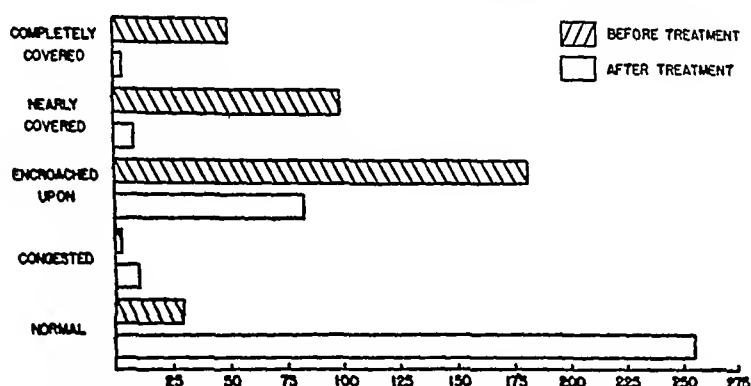


FIG 9 Chart showing given degree of interference with eustachian orifices before and after treatment

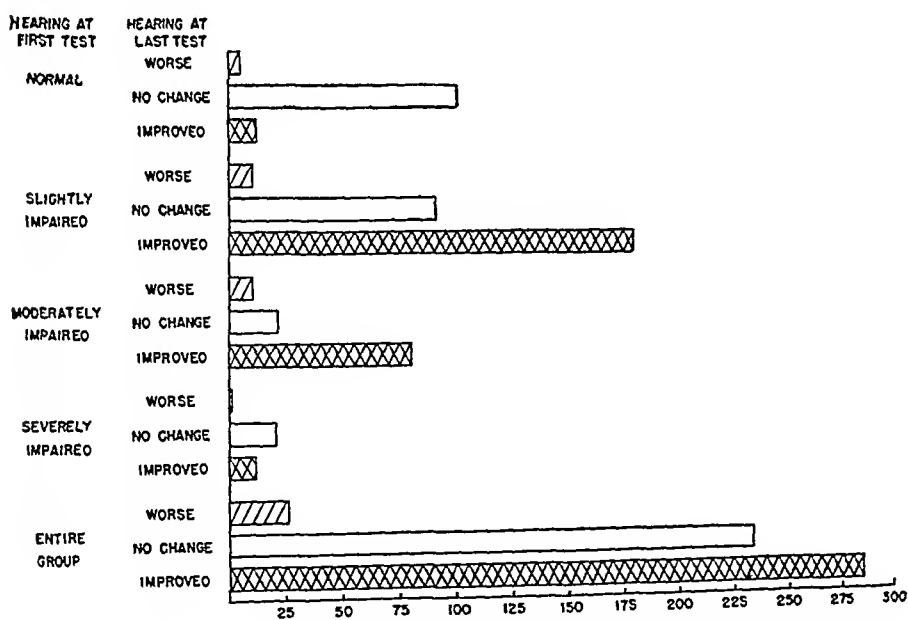


FIG 10 Chart showing given changes in hearing before and after treatment

The change in the appearance of the adenoids and tubal orifices after radium therapy is clearly shown.

The number of children whose hearing improved following irradiation amounts to nearly half of the group with impaired hearing

Figure 12 shows the improvement in symptoms following treatment.

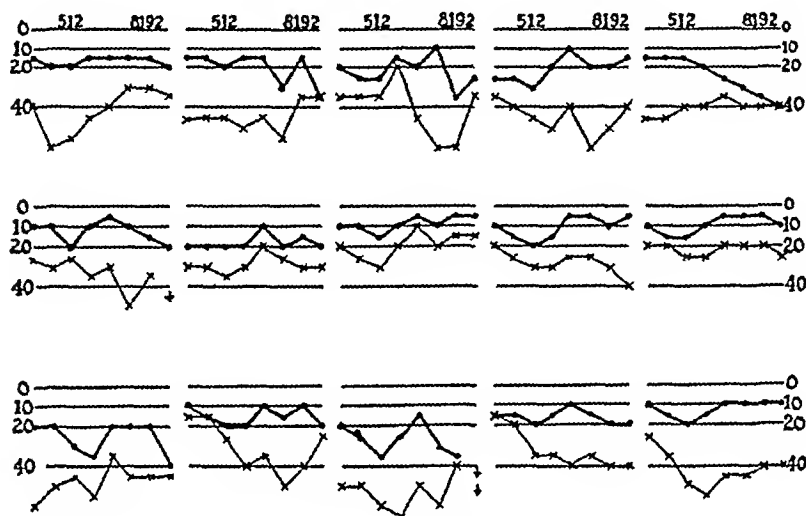


FIG 11 Audiograms showing improvement in hearing after radium treatment. The lower line of each chart represents hearing before treatment

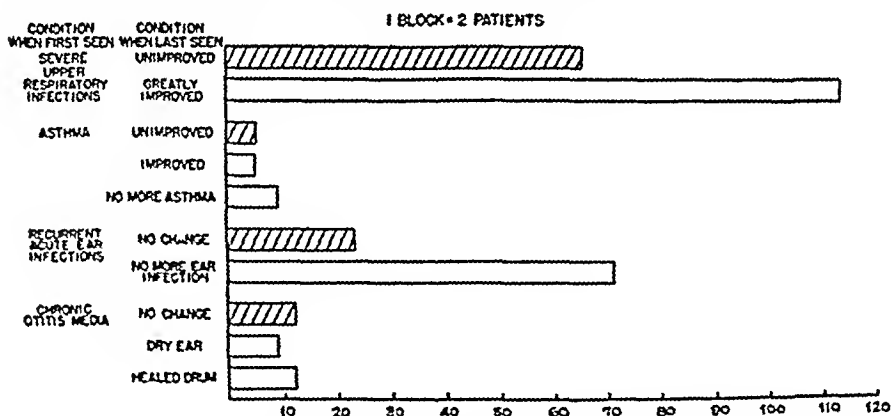


FIG 12 Patients with infections of the upper respiratory tract, asthma, and infections of the ears who showed given results

All of these patients were followed through at least one winter after completion of therapy

Simple local chemotherapy to the nose and ears was also employed in

these patients Surgical removal of lymphoid tissue was carried out in 71 patients during the period of observation In 191 patients lymphoid tissue had been removed surgically prior to their first visit to the clinic

The effect of irradiation on bronchial asthma in children has been difficult to evaluate from the beginning In an attempt to eradicate all

41 PATIENTS WITH BRONCHIAL ASTHMA		WELL	13 (32%)
(AVERAGE FOLLOW-UP OF 35 MONTHS)		BETTER	21 (51%)
		NO BETTER	7 (17%)
MILD ASTHMA	22 (54%)	WELL	9
		BETTER	8
		NO BETTER	5
MODERATE ASTHMA	11 (27%)	WELL	3
		BETTER	6
		NO BETTER	2
MODERATELY SEVERE ASTHMA	1 (2%)	WELL	0
		BETTER	1
		NO BETTER	0
SEVERE ASTHMA	6 (15%)	WELL	1
		BETTER	5
		NO BETTER	0
VERY SEVERE ASTHMA	1 (2%)	WELL	0
		BETTER	1
		NO BETTER	0

FIG 13 Changes in asthmatic patients following irradiation therapy

possible upper respiratory tract infections in the presence of bronchial asthma, thought to be on an infectious basis, irradiation was first tried on an asthmatic about six years ago The dramatic improvement in this child, and in several of the others treated since that time, led to a feeling that there might be a basic relationship between infected adenoid tissue and certain types of bronchial asthma Careful consideration of other factors has forced us to conclude that in asthma, as in the other conditions described above, irradiation of the nasopharynx

IMPAIRMENT THOUGHT TO BE ATTRIBUTABLE TO	NO PATIENTS	% OF TOTAL
UPPER RESPIRATORY AND EAR INFECTIONS	551	83
UNKNOWN	64	10
CONGENITAL	15	2
NERVE DEGENERATION	14	2
STENOSIS EXTERNAL EAR	7	1
MENINGITIS, ETC	6	0.9
OTOSCLEROSIS	4	0.6
TRAUMA	2	0.5

FIG 14 Chart showing estimated etiology of hearing impairments (patients seen in Washington County Clinic)

APPROXIMATE LEVEL IN BEST EAR, PURE TONE AUDIOMETRY	% OF TOTAL PATIENTS	
	FIRST AUDIO	WHEN LAST SEEN
0-20 DECIBEL LOSS	28.6	63.0
25-30	48.9	20.3
35-50	17.1	6.5
55 OR MORE	3.4	3.5
TOTAL LOSS	0.7	0.7
LOSS ONLY ON 8192 OR ABOVE	1.3	6.0

FIG 15 Hearing of patients seen at the Washington County Clinic at initial test and when last seen (best ear)

must be considered as an auxiliary, even though a uniquely helpful auxiliary, to other forms of treatment. Infected adenoid tissue is an important factor in bronchial asthma, when on an infectious basis, but all other contributory causes must be given due consideration and care.

Of 41 children with bronchial asthma (Fig 13) treated with irradiation of the nasopharynx, the average follow-up was 35 months (nearly 3 years) In most of these patients desensitization therapy and other standard measures had been tried, but in the majority of these children no improvement had occurred prior to the institution of irradiation therapy

22 had only mild asthma, 11 had moderate asthma, 1 patient had moderately severe asthma

Of the entire group, 13 or 32 per cent were well when last seen

21 or 51 per cent were definitely improved

7 or 17 per cent had shown no improvement

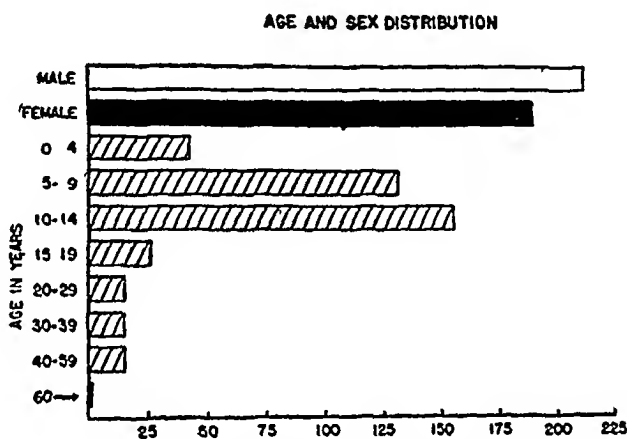


FIG 16 Sex and age distribution of patients seen at the Washington County Clinic

It is interesting to note that more than 83 per cent of all patients seen in this clinic because of hearing impairment had otitis or upper respiratory infections thought to account for the hearing impairment (Fig 14) At the last statistical analysis of this group, carried out during 1947, a comparison was made between the hearing in the better ear at the first examination and the hearing in the better ear when the patient was last seen (Fig 15) Only 28.6 per cent had normal hearing when first seen, but 63 per cent of these children had normal hearing at the last examination Most of these patients had completed therapy a year or more prior to the survey, but a few are still under treatment Before treatment was begun, 66 per cent of these patients had a hearing impairment ranging from a 20 to 50 decibel loss, at the last examina-

		INITIAL AUDIOGRAM										
		A	B	C	D	E	F	G	H	I		
FINAL AUDIOGRAM	A	29	36	17	16	26	3	0	0	0	127	TOTALS FINAL AUDIOGRAM
	B	1	21	4	23	28	6	0	0	0	83	
	C	1	0	2	12	12	2	1	0	0	30	
	D	0	1	1	26	23	3	1	0	0	55	
	E	0	0	0	2	33	4	1	0	0	40	
	F	0	0	0	0	3	11	0	0	0	14	
	G	0	0	0	0	0	2	4	4	0	10	
	H	0	0	0	0	0	0	0	1	0	1	
	I	0	0	0	0	0	0	0	0	1	1	
		31	58	24	79	125	31	7	5	1	361	GRAND TOTAL
		TOTALS INITIAL AUDIOGRAM										

FIG 17 Decibel thresholds

one ear

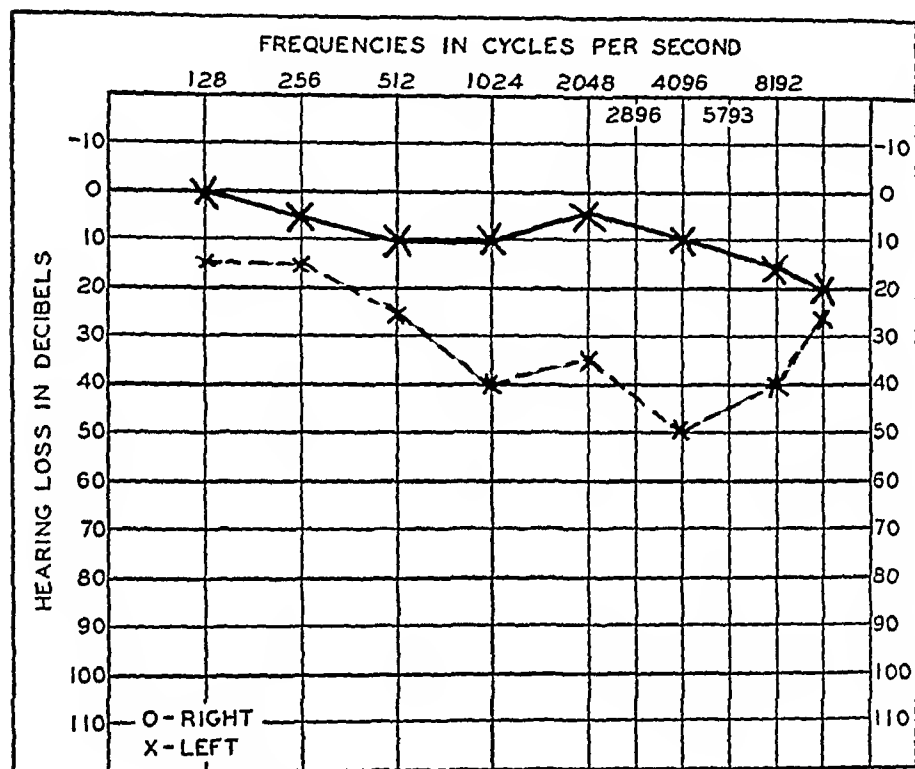
other ear

- A—All tones better than 25
 B—No three poorer than 20, none poorer than 30, exception 8192 or above
 C—No five poorer than 20, none poorer than 30, exception 8192 or above
 D—At least five or more poorer than 20 or one or two poorer than 30, from here up to total or almost total loss
 E—At least five poorer than 20 or one or two poorer than 30, from here on up to total or almost total loss
 F—At least five poorer than 30, may be total or almost total loss
 G—At least three poorer than 50, may be total or almost total loss
 H—Five or more poorer than 50 up to
 I—Total or nearly total loss

- All tones better than 25
 No three poorer than 20, none poorer than 30, exception 8192 or above
 No five poorer than 20, none poorer than 30
 No three poorer than 20, none poorer than 30, exception 8192 or above
 At least three poorer than 20, maybe one or two poorer than 50, no five poorer than 30
 Five or more poorer than 30, may be one or two poorer than 50
 Three or four poorer than 50
 Five or more poorer than 50
 Total or nearly total loss

An attempt to show changes in hearing following therapy considering both ears. Groups defined roughly as above. Diagonal strip contains patients who underwent no significant change. Those to right of diagonal improved and those to left became worse.

tion, after the completion of the treatments, only a little over 26 per cent of the patients remained in this group. In the very severe hearing impairments, such was not the case, 3.4 per cent of the patients were in this group when first seen, and 3.5 per cent when last seen. The charts show the age distribution of these patients (Fig. 16)

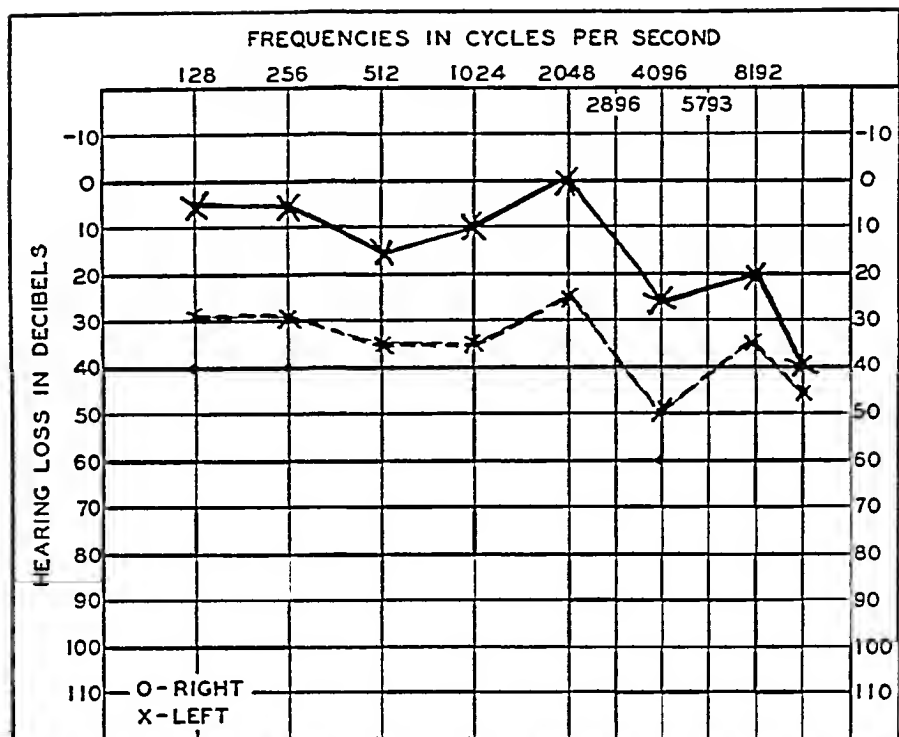


----- 1/29/44 ——— 11/15/46 L M, MW-10

FIG 18 L M, MW-10 First seen with acute otitis media, lt Later given two irradiations of the nasopharynx—total of 66 gm minutes Last seen 8/8/47

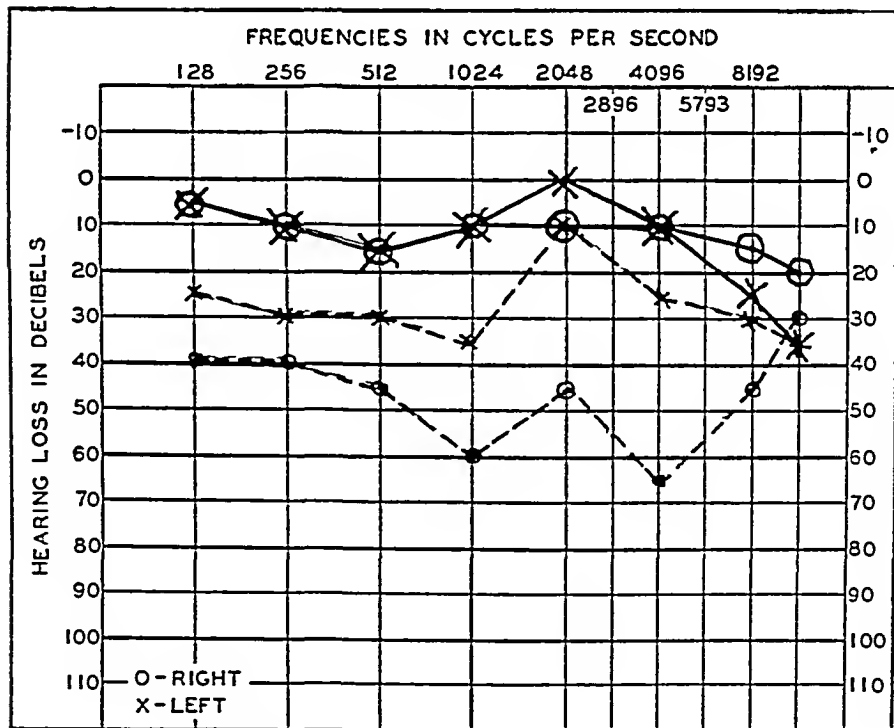
A breakdown of 361 patients, taking into consideration the hearing in both ears, shows that only 11 patients had any increase in their hearing impairment (Fig. 17)

In contrast with this, 26 patients, who at the original examination were classified as moderately severe to very severe impairment in one ear and at least moderate impairment in the other ear, had perfectly normal hearing in both ears at the last examination, after the course



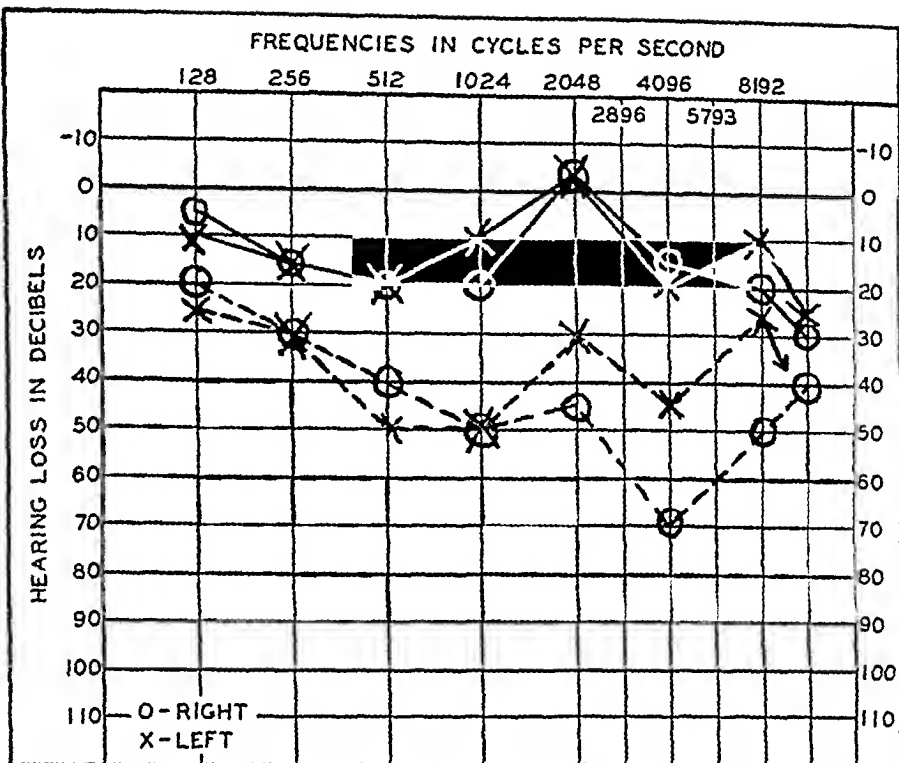
----- 11/15/45 ——— 10/4/47 JB, FW-11

FIG 19 J B, FW-11 Chronic otitis media, lt Six irradiations of the nasopharynx—total of 2 54 gm minutes When last seen, 1/24/48, dry ear



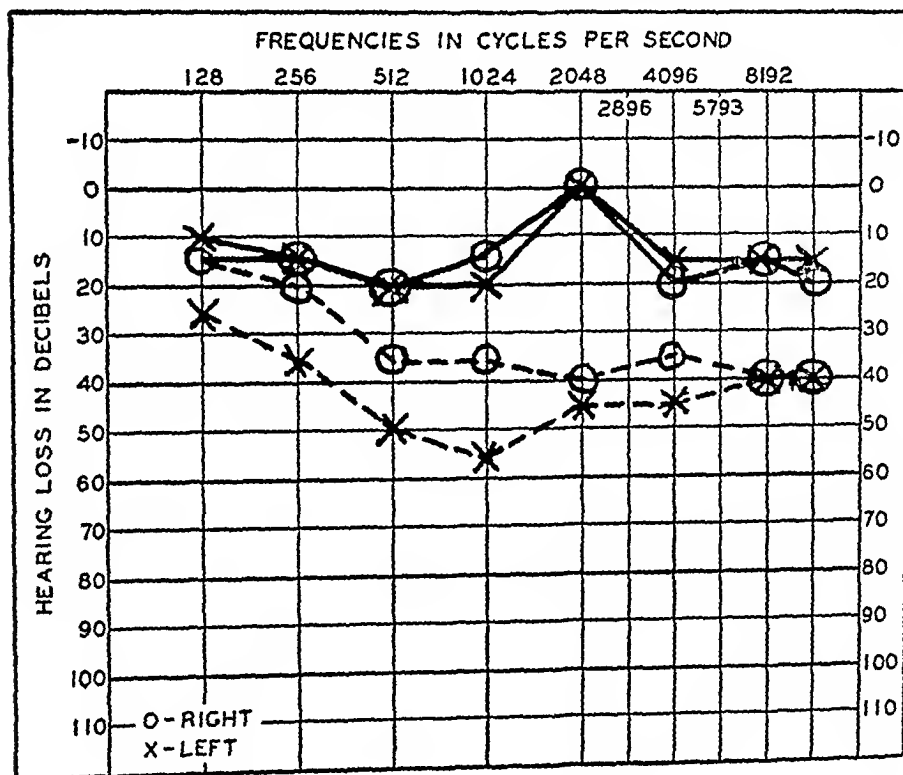
----- 4/8/44 ——— 6/6/46 JB, FW-15

FIG 20 J B, FW-15 Two irradiations of the nasopharynx—total of 66 gm minutes Last seen 7/26/47



----- 1/6/45 ——— 11/15/47 W B, MW-15

FIG 21 W B, MW-15 Chronic otitis media, rt 5 irradiations of the nasopharynx—total of 1 99 gm minutes Ear healed when last seen 11/15/47



----- 7/22/43 ——— 9/24/46 B B, FW-13

FIG 22 B B, FW-13 Six irradiations of the nasopharynx—total of 2 03 gm minutes Last seen 9/28/46

of treatments had been completed. Examination of the accompanying chart will give further data on this analysis.

It is important to note that in this group, as in all of the other groups under our observation, there has never been any complication attributable to the radium treatment. This work covers five years of study and a careful follow up was carried out on nearly every patient.

The changes in hearing are frequently striking enough to excite enthusiasm in both the patient and physician. A few such patients are illustrated in the figures below (Figures 18, 19, 20, 21, 22). These are all children seen and treated in the Hagerstown Clinic.

SUMMARY AND CONCLUSIONS

1 The nasopharynx is the most vulnerable part of the upper respiratory tract. Crypts in nasopharyngeal lymphoid tissue provide the most favorable location for the common cold virus and pathogenic bacteria which cause upper respiratory infections and their complications.

2 The otolaryngologist, by treatments that reduce the size of the lymphoid nodules and the depth of the crypts, can make local conditions unfavorable to recurrent upper respiratory infections.

3 a) In children, surgical removal of adenoids often is followed by recurrence of lymphoid tissue in the nasopharynx and recurrence of undesirable symptoms.

b) Nasopharyngoscopic examination shows that hyperplastic lymphoid nodules are almost invariably present in the lateral walls of the nasopharynx a few months after surgical removal of adenoids in children. Recurrent lymphoid tissue in the nasopharynx should always be sought for and treated in patients with recurring upper respiratory disease, recurring attacks of otitis media, chronic otitis media, recurring attacks of sinusitis, or impaired hearing due to chronic interference with the function of the eustachian tubes.

4 Adenoid tissue is so sensitive to irradiation that doses, so small as to eliminate all possibility of danger, in most instances are sufficient adequately to reduce the size of lymphoid nodules and markedly to decrease their tendency towards hyperplasia.

5 Studies of patients so treated have demonstrated that the following results may be expected in the majority of patients:

a Improvement in hearing or cessation of progressive impairment

when the symptoms are due to interference with the function of the eustachian tubes

b Marked decrease in the number and severity of upper respiratory infections, including acute infections in the sinuses, ears and tonsils

c Improvement in many patients, especially children, suffering from bronchial asthma, when the asthma is on an infectious basis and when other forms of therapy such as desensitization are also employed

6 Irradiation of adenoid tissue has now been used for 24 years and many thousands of patients have been treated. In all this time not a single instance of burn or other complication due to the use of radium has been observed

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RENAL FUNCTION BEFORE AND AFTER SURGICAL RESECTION OF COARCTATION OF THE AORTA¹

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KING in 1926 (1), in his study of four cases of coarctation, confirmed the clinical findings of Wernicke in 1875 and insisted then, and later in 1937 (2), on the presence of arterial pulsations and systolic murmurs in the interscapular region for the diagnosis of coarctation

In 1928, Abbott (3), in her remarkable statistical study of 200 recorded cases with autopsy, did most to elucidate the clinicopathological picture of coarctation, and expressed the opinion that an elevation of the blood pressure in the arms with a lowered or absent blood pressure in the legs was probably the most important single sign of the presence of the obstruction in the thoracic aorta

In 1933, Lewis (4) studied nine cases in great detail and insisted on the necessity of taking the femoral pulse, which in those patients is always either absent, or weak and delayed. Bonnet had emphasized this 30 years before (6). He described two important features of the radiological silhouette of the heart: the increased breadth and density of the shadows of the basal vessels which is associated with their dilatation, and the indistinct shadow of the aortic arch in the left oblique position.

More recently, Campbell and Suzman (5) described a new sign elicited by making the patient stoop forward with his arms hanging down vertically. In this position the collateral arteries in the back

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become very prominent and easily visible on ordinary inspection Campbell and Suzman insisted, as many have done before, on the radiological evidence of notching of the ribs as almost pathognomonic of coarctation

Since these studies, the diagnosis of coarctation of aorta of the adult type has been made much more frequently during the life of the patients afflicted with this congenital (6) anomaly. The statistical proportion of cases of coarctation of the aorta found at autopsy is approximately 1 per 1500 cases (7), being 4 per 10,000 in Lachte's series, 18 per 22,316 in Fawcett's series, 1 per 4500 in Jaffe and Sternberg's series, 16 per 21,481 in Meixner's series, 4 per 1,000 in Hansteen's series (cited by Blackford (8)), 26 per 19,217 in Evans' series (9)

In recent years, cardiovascular surgery has made tremendous progress due to a better knowledge of the physiology of the body hemodynamics, animal experimentation, and improvement in techniques. Since the pioneer experimental work of Blalock and Park (10), Crafoord (11), Gross (12), Blakemore (13), and since the first successful human case in which the constriction was resected and an end-to-end anastomosis was performed by Crafoord of Sweden in October, 1944, more and more attention and interest have been given to this vascular malformation and more cases have come to surgery for correction and relief.

The purpose of this study was to determine the renal circulatory changes before and after operation and to try to interpret them in the light of new experimental concepts of hypertension (14)

The subjects used for this investigation were the first 17 consecutive patients who came to the Johns Hopkins Hospital for surgical correction of coarctation of the aorta. The diagnosis was established by a careful and detailed examination, x-rays of the chest, direct brachial and femoral artery pressures, and angiocardiograms. Fifteen of these patients were operated upon, and the diagnosis was confirmed at operation.

METHODS

The glomerular filtration rate was determined either by the inulin or by the thiosulfate clearance. It has been shown that both the clearance of inulin and of thiosulfate given in a single injection, or in an

infusion at a constant rate, measure the amount of plasma filtered through the glomeruli (15, 16)

The clearance of para-aminohippuric acid (PAH), or of its acetyl derivative para-aminoacetylhippuric acid (PACA), was used to determine the effective renal plasma flow. Newman has shown in dogs and men that the PACA clearance, after a single injection, remains constant at low plasma concentrations (17)

For patients 1, 2 and 8 a single injection of PACA and thiosulfate was given before and after the operation. Single injections of inulin were given preoperatively to cases 4, 5 and 15. Otherwise, PAH and thiosulfate were given as a priming dose, followed by an infusion maintained at a constant rate of 40 drops/minute. The amount given in the priming dose and in the infusion was the same in the pre- and post-operative studies for the same patient. The dosages were calculated to maintain a blood level of PAH between 1 to 2 mg % and of thiosulfate between 20 to 30 mg %.

All the tests were done in the morning, the patient fasting for more than 12 hours and lying in bed. All the patients were catheterized and great care was given to that procedure. Only after the return equalled the amount of distilled water injected in the bladder through the catheter was the test begun. Four or five blood samples and three to five urine specimens of 13 to 20 minutes were taken. At the end of each period the bladder was washed twice with 20 cc of sterile distilled water and these washings were added to that period.

In each of these experiments, the average of the determinations of the renal filtration rate, the renal blood flow (renal plasma flow $\times \frac{100}{100 - \text{hematocrit}}$) and the filtration fraction were corrected to 1.73 square meter surface area.

SUBJECTS

Of these seventeen patients, twelve were males and five were females. All of them were of the white race. The age of the patients is recorded in Table 2. Twelve were studied pre- and postoperatively. Two died during the operation and one a few days later. Two were not operated upon. Of the twelve cases who underwent surgery, ten had a resection of the constricted segment of the aorta and an end-to-end anastomosis,

TABLE 1

Detailed analysis of the renal function studies and their correlation with the age, and the cuff arm and leg blood pressure in the 17 patients with coarctation of aorta

CASES	AGE	S	A	BEFORE OPERATION					AFTER OPERATION					
				Cuff B P		RFR	RBF	PF	Cuff B P		RFR	RBF	PF	
				Arms	Legs				Arms	Legs				
<i>Males</i>														
1 W B , 442140	23	1	9	200/120		0 116	960	0 231	180/88	140/ 90	148	1050	0 245	
2 C O C , 442408	13	1	35	170/ 80		0 141	846	0 278						
3 W D , 424479	18	1	58	185/ 90		0 128	1000	0 236	140/80	130/ 90	112	1050	0 164	
4 C D , 384376	27	1	92	180/ 80		0 100	940	0 203	208/90	140/110	154	1270	0 232	
5 W R H , 381615	24	1	80	170/100		0 99	785	0 280	130/70	154/100	103	1220	0 167	
6 W H , 430560	20	1	72	180/115		0 103	1070	0 164	125/85	140/ 87	152	925	0 279	
7 F K , 441146	18	2	17	159/ 85		0 133	885	0 272						
8 D P , 441762	11	1	41	125/ 85		0 139	1140	0 210	122/84	108/ 84	139	1060	0 216	
9 C S , 436928	10	1	10	135/ 85		0 85	1310	0 095	120/90	120/ 90	137	2100	0 114	
10 J W , HLH 55559	7	0	82	140/ 90	?	110	138	513	0 438					
11 A W , 422617	24	1	98	165/ 90	?	140	138	717	0 37	155/85	132/ 96	126	1080	0 195
12 E L W , 422084	12	1	40	150/ 80		0 160	1170	0 25	135/95	150/100	154	1245	0 213	
Average						122	945	0 252			138	1220	0 201	
<i>Females</i>														
13 B C , 425369	20	1	63	150/100		0 111	920	0 215	130/75	88/ 70	171	1130	0 269	
14 A H , 388545	34	1	49	220/180		0 122	950	0 240						
15 D H , 383369	22	1	55	220/110		0 98	550	0 300						
16 M O , 422966	12	1	40	130/ 85		0 192	1120	0 305	105/65	120/ 80	167	1380	0 216	
17 H S , 440658	14	1	76	195/100		0 139	806	0 326	115/80	125/ 85	146	1020	0 208	
Average						132	869	0 277			161	1176	0 228	

COARCTATION OF THE AORTA - 12 CASES

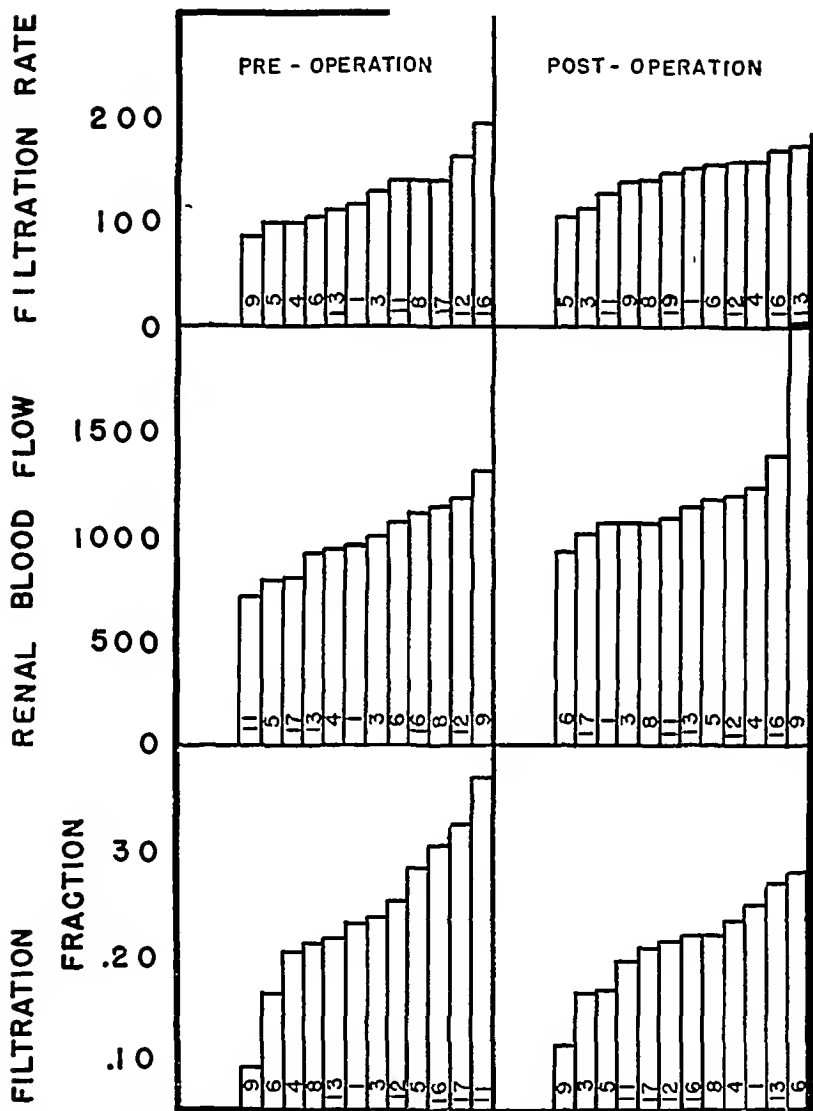


FIGURE 1 Pre- and post-operative determination of the renal filtration rate, the renal blood flow and the filtration fraction in the 12 operated cases

two (No 4 and No 13) had an anastomosis of the left subclavian artery to the distal aorta

RESULTS

Table 1 and Figure 1 show the results of the renal filtration rate (RFR), the filtration fraction (FF), the renal blood flow (RBF), and

of the auscultatory blood pressure in the arms and the legs before and after the operation in the twelve cases successfully operated upon and in the remaining five who either were not operated upon or died during the surgical procedure

A change in the renal filtration rate, the renal plasma flow and the filtration fraction of less than $\pm 10\%$ was not considered significant. Postoperatively, the renal blood flow and the glomerular filtration rate were higher and the filtration fraction tended to decrease as shown in Table 2

TABLE 2
Changes after Operation 12 Cases

This table shows the changes in the filtration rate, the renal blood flow, the filtration fraction and the arm and leg blood pressure after the operation. A change of less than 10% was not considered of any significance

	INCREASE MORE THAN 10%	DECREASE MORE THAN 10 %	NO CHANGE LESS THAN 10%
R Filtration Rate	5	2	5
R Blood Flow	7	1	4
Filtration Fraction	4	6	2
Cuff B P			
Arms			
S	1	8	3
D	2	7	3
Legs			
S	12		
D	12		

DISCUSSION

The preoperative findings confirm those of Friedman et al (18) who first demonstrated a diminution of the renal blood flow in all of their six cases and the presence of a compensatory efferent arteriolar constriction resulting in a nearly normal filtration rate. It is essentially the same pattern as shown by Goldring and Chasis (19) in essential hypertension. The postoperative results also show that in the majority of patients the surgical procedure increases the renal blood flow and relieves the constriction of the efferent artery. Thus the filtration fraction falls.

After operation, one patient (case 6) showed a decreased renal blood

flow and four patients (cases 1, 3, 8 and 13) showed an insignificant change (less than 10%). It is possible that this might be due to the fact that these patients were young (between 10 and 25 years of age) and that anxiety, their considerable apprehension of the catheterization, and their fear of the repeated venipunctures would have been sufficient to bring a decrease in the renal blood flow. Smith has shown in his *Lectures on the Kidney* a beautiful example of this situation (20) in which a hypertensive patient became alarmed in the course of the experiment and his renal blood flow dropped 50%.

It is impossible, even by the careful analysis of our data, to draw a definite conclusion on the nature of the hypertension in most of the cases of coarctation (adult type) of the aorta. Depending probably on the age at which they are seen, these cases can be divided on the basis of the clinical studies of King, Blackford, Abbott and others into three groups: the normotensive group, the systolic hypertensive group, and the systolic-diastolic hypertensive group. The first one does not raise any problem. The systolic hypertension in the second group, (the diastolic pressure being within normal limits) is evidently due to the mechanical obstruction of the constriction of the aorta and to the inability of the large arteries proximal to the coarctation to dilate any further to accommodate the quantity of blood ejected from the left ventricle at each systole. In those cases, normal diastolic pressure indicates that the peripheral resistance is not increased. In many cases of coarctation the cardiac index is within normal limits (21, 22), so that the systolic pressure in the large arteries of the upper extremities will be elevated because of the considerable diminution of the immediate vascular area in which the blood is ejected from the left ventricle and the inability of the large arteries of the upper extremities to distend any further. Bell (25) states that this situation is in some respect similar to aortic stenosis where the obstruction produces left ventricular hypertrophy and intra-ventricular hypertension. Another factor may also play a part, as illustrated in the Figure 2 showing the follow-up of the arm blood pressure in one patient before and after operation. This patient, because of the presence of severe arteriosclerosis with calcareous deposits in the aorta, had an anastomosis of the left subclavian artery to distal aorta as had been done successfully in experimental animals by Blalock and Park (10). Despite a marked increase

in renal blood flow after operation, he presented a further elevation in systolic pressure three and a half months later

The hypertension in the systolic-diastolic group (the largest group), in which the arm diastolic and the systolic pressure is above normal, seems to result from a combination of the factors described above with a humoral mechanism. It is possible that a relative renal ischemia or a diminished pulse pressure in the kidneys would produce a situation analogous to the hypertension produced by the Goldblatt kidney

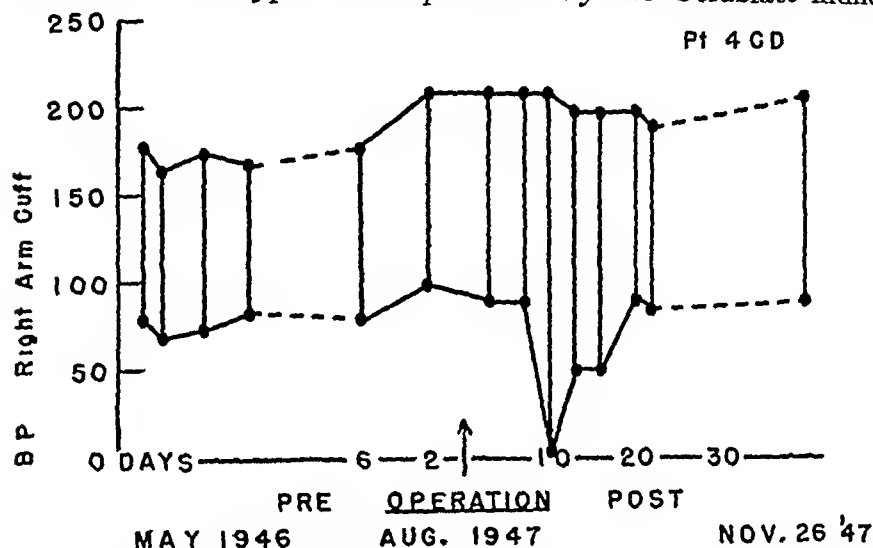


FIGURE 2 This is a follow-up of the cuff arm blood pressure in a patient (CD) who, because of severe arteriosclerosis of the aorta, had an anastomosis of the left subclavian artery with the aorta distally to the constriction

There is much indirect evidence to show the peripheral resistance in such patients is increased and that this increase is generalized. It has been shown by Steele (29) that the femoral mean pressure obtained by the direct method is frequently elevated. Graybiel et al (25) could not find any change in the vessels themselves in studying the arterioles of the muscles and the skin from the arm and the leg from five cases of coarctation. The experimental work done on rats by Rytand (26) and on dogs by Page (27), Brochner (28), Steele (29) and Goldblatt (30) seems to indicate that, in animals, the hypertension produced in the upper and lower extremities by constricting the aorta above the renal arteries is of renal origin.

The present study shows that, in most cases, there is a relationship between the renal blood flow and the blood pressure, but the nature of

this relationship cannot be determined At any rate, the renal circulatory changes and the hypertension are completely reversible

CONCLUSION

Seventeen consecutive patients with coarctation of the aorta of the adult type were studied for their renal filtration rate and blood flow Twelve of these were also studied postoperatively

The surgical resection of the constricted segment and the end-to-end anastomosis of the aorta resulted in most of the patients in an increase of their renal blood flow and a decrease in the filtration fraction

The blood pressure and the renal ischemia in coarctation of the aorta can be completely normal after relief of the aortic constriction

We are deeply indebted to Dr Alfred Blalock and the Department of Surgery for the opportunity to study these patients and use their records

Other hemodynamic studies in these patients will be reported in the *Annals of Surgery*, by Blalock, Bing and their co-workers

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PHYSIOLOGICAL STUDIES IN CONGENITAL HEART DISEASE

VI ADAPTATIONS TO ANOXIA IN CONGENITAL HEART DISEASE WITH CYANOSIS¹

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Patients with the cyanotic type of congenital heart disease and individuals at high altitude show physiological changes attributable to a deficiency of pressure at which oxygen is transferred from capillary blood to the tissues. Barcroft has advanced the concept that acclimatization to high altitude consists of a series of adaptations which tend to alleviate this deficiency by attempting to restore tissue oxygen pressure toward normal sea level values. This, he suggests, is mainly accomplished by increased pulmonary ventilation, polycythemia, and a shift of the oxygen dissociation curve to the left (1).

In recent studies performed on normal adults in the low pressure chamber, Houston and Riley found that their subjects were likewise able to raise the oxygen pressure of the tissues toward normal sea level values. This was accomplished by reducing the difference between the oxygen pressure of inspired air and mean capillary blood. Increased pulmonary ventilation and the shape of the oxyhemoglobin dissociation curve were the principal factors in accomplishing this. Additional, but less important, mechanisms were cardiac output and the oxygen carrying capacity of the blood (2).

Physiological studies undertaken on patients with the cyanotic type of congenital heart disease have shown that in this condition the oxygen saturation of peripheral arterial blood is reduced from birth. At high altitude the lowering of the oxygen saturation in peripheral arterial blood is the result of decreased partial pressure of oxygen in the inspired

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air On the other hand, the cause of anoxemia in congenital heart disease is a reduction in the effective pulmonary blood flow (3) Regardless of the initiating factor, the oxygen saturation and the partial pressure of oxygen of arterial blood are decreased in normal individuals living at reduced barometric pressure and in patients with congenital heart disease and cyanosis Thus, the adaptive processes in both conditions must be directed toward the same goal, the restoration of the tissue oxygen pressure to normal The present investigation consists in a study of these processes in patients with the cyanotic types of congenital heart disease The results of these studies will be compared with those obtained in individuals exposed to decreased barometric pressure

METHODS

Tests were performed on a total of 35 patients with tetralogy of Fallot, studied for diagnostic purposes Reports on the hemodynamics of these individuals have been published in preceding papers (3) The methods and calculations pertaining to the present study will be described in the following paragraphs

The minute volume of ventilation was determined by measuring the quantity of expired air collected in a Douglas bag over periods ranging from $1\frac{1}{2}$ to 3 minutes The oxygen consumed and carbon dioxide produced were determined from analysis of this expired air All values were corrected to dry volume at standard pressure and temperature (STPD) The basal metabolic rate was calculated from the oxygen consumption using the diagram of du Bois (4) Alveolar air was calculated according to a method previously described (5) The blood pH was determined with a Leeds and Northrup shielded glass electrode installed in an incubator regulated to a temperature of 38°C In the presence of a decrease in pCO_2 the carbon dioxide content of serum is predominantly in the form of bicarbonate Therefore, the total carbon dioxide content of serum rather than the serum bicarbonate was determined The partial pressure of carbon dioxide in plasma was then calculated from the Henderson-Hasselbach equation, using the line chart of Peters and Van Slyke (6) The presence of an intracardiac shunt precluded the calculation of effective alveolar gas pressures (7) Consequently, alveolar pO_2 was calculated from the respiratory quo-

tient, the carbon dioxide content of alveolar air and the oxygen content of alveolar and inspired air, using Barcroft's formula (8)

In order to study the oxygen transfer from inspired air to tissue capillaries, it was necessary to determine the partial pressure of oxygen in arterial and in mixed venous blood. This can be accomplished by measuring the pO_2 of the blood directly (9) or by determining it from the oxygen saturation of the blood and the dissociation curve of oxygen. The latter procedure was adopted since in anoxic individuals the values for both arterial and mixed venous blood saturations lie on the steeper part of the dissociation curve. On this portion of the curve the pO_2 can be determined from the oxygen saturation with a much greater degree of accuracy than at the flat upper region. However, the use of the oxygen dissociation curve for the determination of tensions precluded accurate determination of the tensions of oxygen in pulmonary vein blood. The saturation in that vessel had been found to be above 95 per cent. At this high oxygen saturation the pO_2 cannot be accurately determined from the curve. The dissociation curve of individuals with tetralogy of Fallot was obtained by the "in vivo" method of Riley and associates (10). Forty samples were collected from the arterial and venous blood of 35 cyanotic individuals (Fig. 1).

In a study of gradients of oxygen pressure from inspired air to capillary blood, the determination of the oxygen pressure in the latter is of particular significance. Since the capillary pressure cannot be determined directly and varies with anatomical location of the capillary, calculations must be indirect. Barcroft's formula was used.

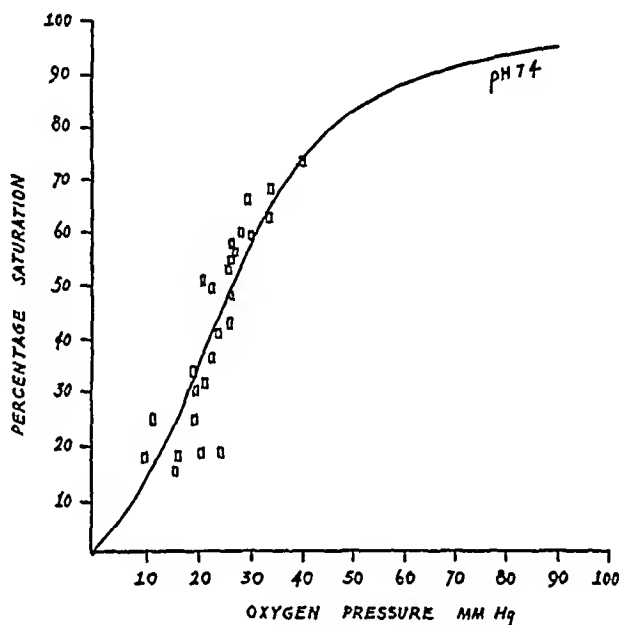
Mean capillary pO_2

$$= \text{mixed venous } pO_2 + \frac{(\text{arterial } pO_2 - \text{mixed venous } pO_2)}{3} \quad (11)$$

The value thus obtained represents, according to Houston and Riley, "the oxygen pressure, which, if it prevailed throughout the entire length of all the capillaries of the body, would not alter the quantity of oxygen diffusing under actual physiological conditions." The normal value for arterial pO_2 was assumed to be 88 mm Hg (saturation 96 per cent), that of mixed venous blood 39 mm (saturation 74 per cent). From these values a normal arteriovenous difference of 49 mm (22 per cent oxygen saturation) and a mean capillary pressure of 55

mm was calculated. Consequently the normal gradient in oxygen pressure from arterial to mean capillary blood equalled 33 mm.

Following Barcroft's example, the transfer of oxygen from inspired air to the tissue capillaries was divided into various gradients. In normal individuals gradients exist between the oxygen tension of inspired air and alveolar air (Barcroft's gradient I), between the oxygen tension of alveolar air and arterial blood (Barcroft's gradient II), and finally



OXYGEN PRESSURE SATURATION POINTS DETERMINED
ON ARTERIAL BLOOD FROM CASES OF CONGENITAL HEART
DISEASE SUPERIMPOSED ON STANDARD CURVE

FIG 1 Grouping of arterial oxygen pressures about a standard dissociation curve

between the oxygen tension of arterial and mean capillary blood (Barcroft's gradient III). In patients with tetralogy of Fallot, however, the presence of an intracardiac right to left shunt introduces an additional gradient between the pO_2 of alveolar air (or, more accurately, the pO_2 of pulmonary vein blood) and of peripheral arterial blood. For reasons outlined above, however, the partial pressure of oxygen in pulmonary vein blood could not be determined with accuracy. Therefore, only the gradient from alveolar air to peripheral arterial blood was calculated.

In evaluating the factors which determine the pO_2 gradient between arterial and mean capillary blood (gradient III) it is necessary to compare this gradient as it exists in the normal at sea level with gradient III in patients of this series. The difference between gradient III in the normal and gradient III in the individuals of this series represents the total gain in this gradient for these patients. This total gain is achieved through the properties of hemoglobin, the oxygen carrying capacity of the blood, and changes in systemic blood flow. In order to determine the relative contribution of each of these factors certain calculations are necessary.

A *The effect of the dissociation curve* The normal arteriovenous difference in oxygen saturation (22 per cent) was subtracted from the arterial oxygen saturation of each individual patient. A venous saturation was thus computed. The partial pressure of oxygen for the observed arterial saturation and for the calculated venous saturation was then determined by reference to a standard oxygen dissociation curve. From these two values the mean capillary pO_2 was calculated. The gradient between arterial pO_2 and the calculated mean capillary pO_2 was then obtained by subtraction. To obtain the gain in gradient III through the dissociation curve alone, the calculated gradient III was subtracted from the normal gradient III.

B *The effect of hemoglobin* The per cent hemoglobin of each individual patient was calculated according to the formula: per cent observed Hgb =
$$\frac{\text{observed oxygen capacity vol per cent}}{\text{normal oxygen capacity (20 vol per cent)}} \times 100$$
 The arteriovenous oxygen difference for the percentage of observed Hgb was then determined according to Barcroft's formula (11)
$$\frac{\text{arteriovenous oxygen difference per cent observed Hg} \times 100}{\text{normal arteriovenous oxygen difference (22 per cent)}} \times 100$$
 percentage observed hemoglobin

This calculated arteriovenous difference in saturation was subtracted from the arterial oxygen saturation of each individual patient. It was then possible to obtain the pO_2 of mixed venous blood, the mean capillary pressure of oxygen, and gradient III, all with reference to the effect of hemoglobin. The gain due to increased hemoglobin was then determined according to the equation: Gain through hemoglobin = gradient III in the normal - (gradient III calculated for Hgb + gain through curve)

C *The effect of the systemic blood flow* The effect on gradient III resulting from changes in the systemic blood flow was calculated according to the formula Effect of systemic blood flow = total gain — (gain through dissociation curve + gain through hemoglobin) Methods involved in the calculation of the systemic flow have been described in detail in a preceding publication (12)

General technique All blood oxygen and carbon dioxide contents were determined by the manometric method of Van Slyke and Neill (13) The oxygen capacity of blood was obtained by equilibration of 8 cc of blood with room air in tonometers of 350 cc capacity The blood was collected in syringes under oil and transferred under oil into bottles of 20 cc capacity containing 2 mgs sodium fluoride and 15 mgs potassium oxalate The oxygen tension was determined by the method of Riley et al (9) All gas analyses were performed in the Haldane apparatus

Results and discussion *The oxygen dissociation curve of hemoglobin* Figure 1 demonstrates that the partial pressure of oxygen obtained on a series of 16 patients with tetralogy of Fallot cluster around the standard oxygen dissociation curve of hemoglobin As the pH of these samples was close to 7.4, the points determined on the curve express the true relationship between the partial pressure of oxygen and the oxygen saturation The use of a standard oxygen dissociation curve is therefore valid This observation is at variance with the findings of Barcroft on subjects at high altitude He found a shift of the oxygen dissociation curve to the left in natives living high in the Andes (11) On the other hand, Houston and Riley (2), as well as Keys (14), noticed a shift of the oxygen dissociation curve to the right in individuals exposed to low oxygen pressures

Pulmonary ventilation and respiratory gas exchange Table I presents the results in the determination of the minute volume of respiration, the oxygen uptake, and the carbon dioxide output In 29 out of 30 patients the minute volume of respiration was increased above normal In the absence of an intracardiac shunt an increase in respiratory minute volume lessens the gradient between the oxygen tension of inspired air and capillary blood by reducing the difference between alveolar and arterial pO_2 In individuals with tetralogy of Fallot, on the other

hand, the effect of hyperventilation is largely offset by the presence of the right to left intracardiac shunt (Fig 3)

TABLE I

NAME	SURFACE AREA M ²	LV/MIN / M ² *	R Q	CC/MIN O ₂ CONS	CC/MIN CO ₂ OUTPUT	BMR
V S	1 68	5 5	89	241	218	+9
D M	0 83	3 6	87	95	85	-32
D S	1 30	3 4	83	138	117	-18
O J	1 80	2 5	90	162	152	-30
M C	1 28	4 1	88	161	143	-5
L S	1 68	3 0	88	172	153	-23
C H	1 43	3 2	1 0	119	119	-34
R A	1 46	4 7	1 0	156	157	-25
G S	1 50	2 7	1 08	103	113	-48
R C	1 35	6 1	1 26	162	207	-24
A P	1 30	3 7	82	131	109	-23
L P	1 59	3 3	81	164	134	-19
J H	1 54	5 6	82	117	147	-27
A F	0 66	9 5	90	126	115	-8
J O	1 68	2 4	73	180	153	-27
V A	0 76	6 1	83	105	91	-26
D F	1 34	5 0	1 08	150	165	-33
J F _f	0 95	3 4	90	77	72	-48
J F _m	1 70	4 7	1 10	211	241	-22
E R	1 43	3 5	1 08	112	122	-39
B H	0 96	7 3	1 18	111	132	-28
D B	0 77	7 5	1 02	95	97	-24
V B	1 01	6 3	87	123	107	-24
R W	1 50	7 8	1 18	195	231	-15
R F	1 02	7 9	88	161	142	-1
J M _f	0 82	5 1	98	91	90	-4
A S	1 59	3 9	89	148	131	-26
A OC	0 93	4 5	83	96	80	-41
S C	1 50	4 6	1 03	135	140	-46
E L	1 44	5 2	1 01	215	220	+18

* Normal at 0° and prevailing atm p = 2 82

Of special interest is the reduction of the oxygen consumptions and consequently of the basal metabolic rate In 28 of 30 patients the basal metabolic rate was either markedly reduced or at the lower limit of normal In one individual (G S) in whom one pulmonary artery

was absent, a value of -45 was determined. In two patients of this series (E L, V S) the basal metabolic rate was positive. These findings suggest that metabolic adjustments take place which keep the tissue oxygen tension at the lowest possible level. In contrast, Houston and Riley among others have shown that the oxygen consumption of normal individuals remained the same at high altitude as at sea level. Their observation implies that cellular metabolism remains normal as the barometric pressure decreases (2).

The acid base balance The findings concerning the hydrogen ion concentration, the serum carbon dioxide, the partial pressure of carbon dioxide, and the serum chloride of 21 patients are illustrated in Figure 2. It may be seen that the pH was within normal range. The values for total carbon dioxide, on the other hand, were significantly reduced. Since the pH varies directly with the bicarbonate and inversely with the $p\text{CO}_2$, the reduction in serum carbon dioxide was accompanied by a decrease in the $p\text{CO}_2$. Serum chloride (Fig 2) and blood lactic acid values were within normal limits (15). The assumption might be ventured that the failure of the pH to rise as the $p\text{CO}_2$ fell is the result of increased elimination of bicarbonate. These results contrast with those obtained by a series of investigators on normal individuals at high altitude. Keys, for instance, demonstrated increased alkalinity of the blood in persons living at altitudes ranging from 4,000 to 12,000 feet, above this altitude he noticed either no further change or a return to values observed at sea level (16). Dill and associates found that the pH rose as the partial pressure of oxygen in the inspired air decreased (17). Similar results were obtained by Houston and Riley in the low pressure chamber (2).

The oxygen transfer system Changes in the oxygen transfer system from inspired air to mean capillary blood are illustrated in Figure 3. In the patients of this series the average gradient between the pressure of oxygen in inspired and alveolar air (gradient I) is 25 mm of Hg. This represents a gain of 32 mm Hg since in the normal at sea level gradient I equals 57 mm. At altitudes of 20,000 feet the reduction in gradient I is of similar magnitude. This reduction in gradient observed in both conditions is the result of increases in respiratory minute volume.

The average drop in $p\text{O}_2$ from alveolar air to arterial blood (gradient

II) in individuals with congenital heart disease equals 57 mm Hg (Fig. 3) In the normal individual at sea level this gradient is 5 mm and at altitudes of 20,000 feet it is 2 mm (2) The large difference

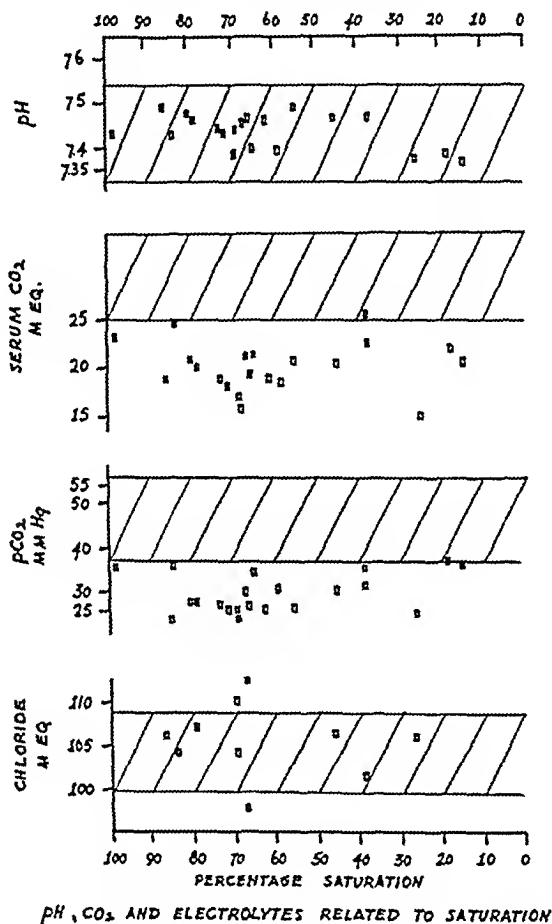


FIG 2 Relationship of pH, CO₂, electrolytes, and saturation in patients with congenital heart disease. Shaded areas indicate normal range. Serum CO₂ and pCO₂ are decreased proportionately. As a result the pH remains within normal range.

in this gradient between the normal and patients with the cyanotic type of congenital heart disease is the result of the intracardiac right to left shunt in the latter. The shunt lowers the oxygen tension of arterial blood and it is because of this that hyperventilation loses its effect in the patients of this series. Consequently a diminution of the

oxygen gradient between the arterial and mean capillary blood_s (gradient III) remains the only means of restoring the tissue oxygen tension toward normal

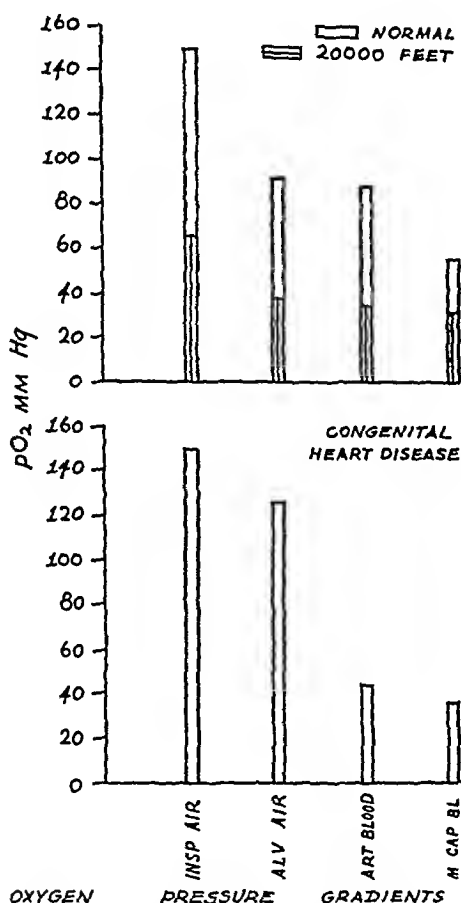


FIG 3 Gradients in the oxygen transfer system from inspired air to capillary blood in normal individuals at sea level and at high altitude, and in patients with congenital heart disease

It has been shown that gradient III may be lowered by the integrated action of three major factors. The first of these is the shape of the oxygen dissociation curve of hemoglobin. The shape of this curve is such that on its steeper portion a given drop in saturation produces a much smaller fall in oxygen tension than at its flat upper part. This fact is of major importance in reducing gradient III at high altitude.

where the points for arterial oxygen saturation lie on the steep part of the curve

TABLE II .
Effect due to Curve

NO	NAME	% ART SAT	% VENOUS SAT CALCUL	OBS ART PO ₂	CALC. MIXED VENOUS PO ₂	ΔPO ₂	CALC MEAN CAP PO ₂	CALC GRADIENT III	GAIN DUE TO CURVE
1	V S	60.6	38.6	32.0	21.5	10.5	25.0	7.0	26.0
2	D M	79.8	57.8	45.0	30.0	15.0	35.0	10.0	23.0
3	D S	63.8	41.8	33.3	23.0	10.3	26.4	6.9	26.1
4	O J	75.0	53.0	40.5	28.0	12.5	32.2	8.3	24.7
5	M C	69.0	47.0	36.0	25.0	11.0	28.7	7.3	24.7
6	L S	71.5	49.5	38.0	26.2	11.8	30.1	7.9	25.1
7	C H	60.8	38.8	31.8	21.8	10.0	25.1	6.7	26.3
8	R A	82.0	60.0	47.8	31.1	16.7	36.7	11.1	21.9
9	G S	77.7	55.7	42.5	29.6	12.9	33.9	8.6	24.4
10	R C	72.2	50.2	38.3	26.5	11.8	30.4	7.9	25.1
11	A P	85.5	63.5	53.0	33.0	20.0	39.7	13.3	19.7
12	L P	79.0	57.0	44.0	29.8	14.2	34.5	9.5	23.5
13	J H	57.4	35.4	30.2	20.0	10.2	23.4	6.8	26.2
14	A F	57.8	35.8	30.4	20.2	10.2	23.6	6.8	26.2
15	J O	80.0	58.0	45.7	30.2	15.5	35.4	10.3	22.7
16	V H	89.0	67.0	60.0	35.0	25.0	43.3	16.7	16.3
17	D F	80.1	58.1	45.7	30.2	15.5	35.4	10.3	22.7
18	J F _f	66.0	44.0	34.5	23.9	10.6	27.4	7.1	25.9
19	J F _m	78.6	56.6	43.6	29.5	14.1	34.2	9.4	23.6
20	E R	84.5	62.5	51.5	32.6	18.9	38.9	12.6	20.4
21	B H	73.4	51.4	39.1	27.1	12.0	31.1	8.0	25.0
22	D B	58.6	36.6	30.5	20.5	10.0	23.8	6.7	26.3
23	V B	75.0	53.0	41.3	28.0	13.3	32.4	8.9	24.1
24	R W	81.0	59.0	46.5	31.0	15.5	36.2	10.3	22.7
25	R F	70.9	48.9	37.3	26.0	11.3	29.8	7.5	25.5
26	J M _f	78.0	56.0	42.5	29.1	13.4	33.6	8.9	24.1
27	A S	74.8	52.8	41.3	27.9	13.4	32.4	8.9	24.1
28	A OC	64.5	42.5	33.5	23.2	10.3	26.6	6.9	26.1
29	S C	91.0	69.0	66.0	36.0	30.0	46.0	20.0	13.0
30	E L	82.5	60.5	48.5	31.5	17.0	37.2	11.3	21.7

The second factor is the increase in the quantity of hemoglobin. According to Haldane and Priestley (18), polycythemia maintains the oxygen saturation and tension of venous blood at a higher level since less oxygen is discharged from each individual red cell. This raises

the mean capillary pressure of oxygen and leads to reduction in gradient III. However, it has been found that at high altitude the additional gain in gradient III due to polycythemia was insignificant (2).

A rise in cardiac output represents the third factor. Increased blood flow through the tissues reduces the difference in the arteriovenous oxygen saturation and thereby raises the mean capillary pressure of oxygen. Houston and Riley found an increase in cardiac output dur-

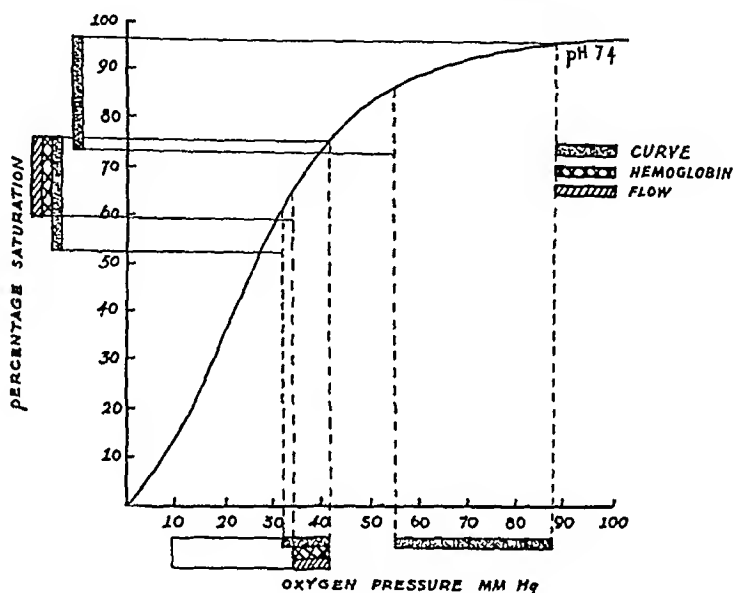


FIG 4 The role of three factors in decreasing the oxygen pressure gradient from arterial to mean capillary blood. The gain over the normal is represented by the unshaded area in the lower rectangle.

ing the first days of exposure to low barometric pressure (2), and Harrison and Blalock demonstrated that anoxia had a similar effect (19). Most investigators agree, however, that the cardiac output returns to normal during the chronic phase of anoxia (20).

An evaluation of the role played by each of these mechanisms reveals that in the patients of this series the shape of the dissociation curve is the most important factor. Table II indicates that because of the characteristics of the curve alone, the gradient between arterial and mean capillary blood equals 9.4 mm, that is, 23.6 mm less than in the normal individual at sea level. It can be seen from Figure 4 that this

factor is primarily responsible for the reduction in gradient III The second factor, the oxygen capacity of blood, is less significant, the aver-

TABLE III
Effect due to Hgb

NO	NAME	OBS CAP VOL. %	HGB GM	% HGB	A-V DIF IN % SAT CALCUL FOR HGB	% OBS SAT	% CALC MIXED VENOUS SAT	OBS ART PO ₂	CALC MIXED VEN- OUS PO ₂	A-V Δ PO ₂	MEAN CAP PO ₂	GRADIENT III REP TO HGB	GAIN
1	V S	34 0	25 5	170 0	12 90	60 6	47 7	32 0	25 7	46 3	27 8	4 2	2 8
2	D M	28 5	21 3	142 5	15 40	79 8	64 4	45 0	33 4	11 6	37 3	7 7	2 3
3	D S	32 0	23 8	160 0	13 70	63 8	50 1	33 3	26 5	6 8	28 8	4 5	2 4
4	O J	32 0	23 8	160 0	13 70	75 0	61 3	40 5	32 0	8 5	34 8	5 7	2 6
5	M C	35 3	26 7	176 5	12 40	69 0	56 6	36 0	29 0	7 0	31 3	4 7	2 6
6	L S	34 0	25 5	170 0	12 90	71 5	58 6	38 0	30 2	7 8	32 8	5 2	2 7
7	C H	26 3	19 6	131 5	16 70	60 8	44 1	31 8	24 0	7 8	26 6	5 2	1 5
8	R A	35 2	26 3	176 0	12 50	82 0	69 5	47 8	36 5	11 3	40 3	7 5	3 6
9	G S	31 5	23 7	157 5	13 95	77 7	63 75	42 5	33 2	9 3	36 3	6 2	2 4
10	R C	31 7	23 7	158 5	13 85	72 2	58 35	38 3	30 3	8 0	33 0	5 3	2 6
11	A P	21 0	15 7	105 0	20 90	85 5	64 6	53 0	41 5	11 5	45 3	7 7	5 6
12	L P	30 3	22 7	151 5	14 50	79 0	64 5	44 0	33 5	10 5	37 0	7 0	2 5
13	J H	39 8	29 7	199 0	11 00	57 4	46 4	30 2	25 0	5 2	26 7	3 5	3 3
14	A F	34 5	25 8	172 5	12 75	57 8	45 05	30 4	24 0	6 4	26 1	4 3	2 5
15	J O	33 4	25 0	167 0	13 15	80 0	66 85	45 7	35 0	10 7	38 6	7 1	3 2
16	V H	20 0	14 9	100 0	22 00	89 0	67 0	60 0	35 0	25 0	43 3	16 7	0
17	D F	24 3	18 2	121 5	18 10	80 1	62 0	45 7	32 3	13 4	36 8	8 9	1 4
18	J F f	31 5	23 7	157 5	13 95	66 0	52 05	34 5	27 3	7 2	29 7	4 8	2 3
19	J F m	26 7	19 9	133 5	16 50	78 6	62 1	43 6	32 3	11 3	36 1	7 5	1 9
20	E R	29 1	21 8	145 5	15 10	84 5	69 4	51 5	36 4	15 1	41 4	10 1	2 5
21	B H	23 3	17 4	116 5	18 90	73 4	54 5	39 1	28 5	10 6	32 0	7 1	0 9
22	D B	30 4	22 7	152 0	14 50	58 6	44 1	30 5	23 8	6 7	26 0	4 5	2 2
23	V B	28 2	21 1	141 0	15 60	75 0	59 4	41 3	31 0	10 3	34 4	6 9	2 0
24	R W	33 0	24 9	165 0	13 35	81 0	67 65	46 5	35 5	11 0	39 2	7 3	3 0
25	R F	34 0	25 5	170 0	12 95	70 9	57 95	37 3	30 2	7 1	32 6	4 7	2 8
26	J M f	24 7	18 3	123 5	17 80	78 0	60 2	42 5	31 5	11 0	35 2	7 3	1 6
27	A S	24 1	18 2	120 5	18 25	74 8	56 55	41 3	29 5	11 8	33 4	7 9	1 0
28	A OC	30 4	22 7	152 0	14 50	64 5	50 0	33 5	26 5	7 0	28 8	4 7	2 2
29	S C	25 5	19 1	127 5	17 30	91 0	73 7	66 0	39 4	26 6	48 3	17 7	2 3
30	E L	27 7	20 8	138 5	15 90	82 5	66 6	48 5	34 6	13 9	39 2	9 3	2 0

age gain amounting to 2.35 mm Hg (Table III) It is of importance, however, to bear in mind that the gain from this factor depends to a

large extent on that derived from the dissociation curve Table III shows that gradient III derived for hemoglobin alone averages 10 mm

TABLE IV
Effect due to Flow

NO	NAME	OBS MIXED VENOUS O ₂ CON- TENT	O ₂ CAPAC- ITY	% SAT MIXED VENOUS BLOOD	OBS ART PO ₂	OBS MIXED VENOUS PO ₂	A-V ΔPO ₂	MEAN CAP PO ₂	OBS GRAD- IENT III	GAIN	SYS- TEMIC FLOW L/ MIN /M ²
1	V S	18.8	34.0	55.2	32.0	29.2	2.8	30.1	1.9	2.3	3.87
2	D M	18.2	28.5	64.0	45.0	33.2	12.8	37.5	8.5	-0.8	2.55
3	D S	17.6	32.0	55.0	33.3	28.8	4.5	30.3	3.0	1.5	3.70
4	O J	20.4	32.0	63.8	40.5	33.0	7.5	35.5	5.0	0.7	2.52
5	M C	22.8	35.3	64.5	36.0	33.5	2.5	34.3	1.7	3.0	8.40
6	L S	22.3	34.1	65.4	38.0	34.0	4.0	35.3	2.7	2.5	4.80
7	C H	14.6	26.3	55.5	31.8	29.2	2.6	30.1	1.7	3.5	5.89
8	R A	23.1	35.2	65.6	47.8	34.2	13.6	38.7	9.1	-1.6	1.84
9	J S	16.9	31.5	53.7	42.5	28.0	14.5	32.8	9.7	-3.5	0.90
10	R C	18.3	31.7	57.8	38.3	30.4	7.9	33.0	5.3	0	2.64
11	A P	13.7	21.0	65.2	53.0	34.0	19.0	40.3	12.7	-5.0	2.24
12	L P	19.8	30.3	65.4	44.0	34.1	9.9	37.4	6.6	0.4	2.38
13	J H	20.1	39.8	50.8	30.2	26.8	3.4	27.9	2.3	1.2	4.20
14	A F	17.5	34.5	50.8	30.4	26.8	3.6	28.0	2.4	1.9	8.20
15	J O	22.9	33.4	68.5	45.7	36.0	9.7	39.2	6.5	0.6	2.97
16	V H	9.6	20.0	48.1	60.0	25.6	34.4	37.1	22.9	-6.2	1.90
17	D F	16.5	24.3	67.9	45.7	35.4	10.3	38.8	6.9	2.0	3.90
18	J F <i>f</i>	16.3	31.5	51.8	34.5	27.2	7.3	29.6	4.9	-0.1	1.75
19	J F <i>m</i>	12.5	26.7	46.7	43.6	25.0	18.6	31.2	12.4	-4.9	1.50
20	E R	18.1	29.1	62.0	51.5	32.2	19.3	38.6	12.9	-2.8	1.23
21	B H	14.8	23.3	63.4	39.1	33.0	6.1	35.0	4.1	3.0	5.16
22	D B	12.1	30.4	39.5	30.5	22.0	8.5	24.8	5.7	-0.8	2.10
23	V B	15.2	28.2	54.0	41.3	28.3	13.0	32.6	8.7	-1.8	7.25
24	R W	20.5	33.0	62.3	46.5	32.6	13.9	37.2	9.3	-2.0	2.06
25	R F	19.3	34.0	56.8	37.3	29.8	7.5	32.3	5.0	-0.3	3.24
26	J M <i>f</i>	18.3	24.7	74.0	42.5	40.0	2.5	40.8	1.7	5.6	11.40
27	A S	13.7	24.1	56.8	41.3	29.4	11.9	33.3	8.0	-0.1	2.18
28	A OC	17.0	30.4	56.0	33.5	29.3	4.2	30.7	2.8	1.9	3.30
29	S C	17.1	25.5	67.1	66.0	35.1	30.9	45.4	20.6	-2.9	1.50
30	E L	19.1	27.7	69.0	48.5	36.2	12.3	40.3	8.2	1.1	4.20

which represents a considerable gain of 23 mm over the gradient III of the normal individual at sea level. Since, however, the effect of the curve has already markedly reduced gradient III, the additional diminution in the gradient caused by the polycythemia is relatively small

Similar considerations pertain to the evaluation of the effect of the systemic flow on gradient III. If the systemic flow is increased above

TABLE V

NAME	ART PO_2 mm Hg	MEAN CAP PO_2 mm.Hg	TOTAL GRADIENT III PO_2 mm Hg	GAIN IN GRADIENT III		
				Curve mm Hg	Hgb mm Hg	Flow mm.Hg
V S	32.0	30.1	1.9	26.0	2.8	2.3
D M	45.0	37.5	8.5	23.0	2.3	-0.8
R S	33.3	30.3	3.0	26.1	2.4	1.5
O J	40.5	35.5	5.0	24.7	2.6	0.8
M C	36.0	34.3	1.7	25.7	2.6	3.0
L S	38.0	35.3	2.7	25.1	2.7	2.5
C H	31.8	30.1	1.7	26.3	1.5	3.5
R A	47.8	38.7	9.1	21.9	3.6	-1.6
J S	42.5	32.8	9.7	24.4	2.4	-3.5
R C	38.3	33.0	5.3	25.1	2.6	0
A P	53.0	40.3	12.7	19.7	5.6	-5.0
L P	44.0	37.4	6.6	23.5	2.5	0.4
J H	30.2	27.9	2.3	26.2	3.3	1.2
A T	30.4	28.4	2.4	26.2	2.5	1.9
J O	45.7	39.2	6.5	22.7	3.2	0.6
V H	60.0	37.1	22.9	16.3	0	-6.2
D T	45.7	38.8	6.9	22.7	1.4	2.0
J T f	34.5	29.6	1.9	25.9	2.3	-0.1
J T m	43.6	31.2	12.4	23.6	1.9	-4.9
E R	51.5	38.6	12.9	20.4	2.5	-2.8
B H	39.1	35.0	4.1	25.0	0.9	3.0
D B	30.5	24.8	5.7	26.3	2.2	-0.8
V B	41.3	32.6	8.7	24.1	2.0	-1.8
R W	46.5	37.2	9.3	22.7	3.0	-2.0
R T	37.3	32.3	5.0	25.5	2.8	-0.3
J M f	42.5	40.8	1.7	24.1	1.6	5.6
A S	41.3	33.7	8.0	24.1	1.0	-0.1
A OC	33.5	30.7	2.8	26.1	2.2	1.9
S C	66.0	45.4	20.6	13.0	2.3	-2.9
E L	48.5	40.3	8.2	21.7	2.0	1.1
Arith. Mean	41.6	34.62	7.12	23.6	2.35	0

normal, a significant gain from this factor may be expected in patients in whom the combined gain from the dissociation curve and the polycythemia is small. Conversely, a loss from this factor may result in patients with small systemic flows in whom the combined gain from the

dissociation curve and the polycythemia is large. The effect of this factor is further limited, since the systemic flow in tetralogy of Fallot is controlled by mechanical factors associated with the cardiac malformation (3). Table IV shows that 15 out of 30 patients show a slight gain, the rest some loss, in gradient. The average effect of this factor is zero.

The gains in gradient III are summarized in Table V.

SUMMARY AND CONCLUSIONS

The adaptive processes to anoxia in patients with congenital heart disease have been investigated. They have been discussed with reference to adaptations of normal individuals at high altitude. In the patients of this series the anoxemia was the result of the intracardiac shunt which increases the gradient of oxygen tension between alveolar air and arterial blood. On the other hand, in normal individuals exposed to high altitude, anoxemia is the result of decreased oxygen pressure in the inspired air.

At reduced barometric pressures, hyperventilation effectively lowers the total gradient of pO_2 between inspired air and mean capillary blood. In the patients of this series the presence of the intracardiac shunt nullified the effect of hyperventilation.

Another major difference was the low oxygen consumption observed in patients with congenital heart disease. The cause of the decrease in the basal metabolic rate is still obscure, it is possible, however, that it is the result of metabolic adjustments to prolonged severe anoxemia. In several patients with tetralogy of Fallot the oxygen consumption rose following the Blalock operation, indicating that the reduction of the metabolic rate may be reversible (21).

The adjustment of the acid base balance to anoxemia may be more effective in the patients of this series than in normal individuals at high altitude. In both cases hyperventilation results in a reduction of the partial pressure of carbon dioxide in plasma. At reduced barometric pressure the carbon dioxide deficit is not entirely compensated by a decrease in bicarbonate, and as a result the pH is elevated. In congenital heart disease with cyanosis, on the other hand, the bicarbonate content of plasma was reduced by an equivalent amount and the pH was maintained at normal values.

The pO_2 gradient from arterial to capillary blood (gradient III) is reduced in the patients of this series and in individuals exposed to high altitude. In both conditions this reduction is mainly the result of the shape of the oxygen dissociation curve and to a lesser extent of an increase in the oxygen carrying capacity of the blood. The systemic flow shows an initial increase in normal individuals exposed to lowered barometric pressure and thereby lowers gradient III.

Despite individual variations in the patients of this series, the average effect of their systemic flows did not alter the gradient.

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BOOK REVIEWS

(These reviews represent the individual opinions of the reviewers and not necessarily those of the members of the Editorial Board of the Bulletin)

Care and Management of Laboratory Animals, The Edited by ALASTAIR N WORDEN Illus 368 pp \$8 50 *The Williams & Wilkins Company, Baltimore, 1947*

This handbook of the Universities Federation for Animal Welfare is an excellent British text which is full of both the everyday useful information, such as how to pick up and hold a rabbit or how to house and feed different species of rats, and more obscure (to us) information such as anaesthesia of hedgehogs. Besides the rabbit, guinea pig, several kinds of rats and mice, there are chapters on voles, hamsters, ferrets, pigeons, canaries, amphibia and freshwater fish, each obviously by an individual who is actively working with the animal in question. A separate chapter on Law and Practice or The Rights of Laboratory Animals is particularly interesting to workers in this country. Herein is indicated the much stronger hold the anti-vivisectionists have on the use of laboratory animals in England than here. Laws and regulations, at least on paper, completely control the use of animals in research. Different classes of animals are established and different classes of investigation are permissible. In a way this chapter is a renewed warning to laboratory workers that the public has a right to insist on the most humane type of care of animals and that we must recognize that right.

It is impossible for one person to review adequately such diversified although concisely arranged information, but the reader may note that, although the publication date is 1947, there is little new information on the use of insecticides in the chapter on "Pests of the Animal House and their Control" by Buxton and Busvine, apparently because this chapter was written in 1944. Thus there is nothing on Gammexane against cockroaches.

There are no chapters on anthropoids, dogs, cats, or poultry, but there are a few scattered notes in the last chapter giving good references.

A conspectus of the Elements of Statistical Analysis is included in this book with the thought that the appreciation of the value of a statistical approach will help the reader to use fewer animals in his experiments. No doubt this is a useful chapter but still seems out of place in a practical handbook.

There is no question but that the excellence of this much needed handbook will make it necessary in all laboratories where small animals are used.

F B B

Identification of Tumors By NATHAN CHANDLER FOOT Illus 397 pp \$6 00
J B Lippincott Co, Philadelphia 1948

The author's preface anticipates the reviewers' comments regarding the discrepancy between the comprehensive implication of the word "identification"

and the brief discussion of human tumors as presented. The book is offered as a "ready reference" book on human tumors. It is short, "systematically compiled", and affords "an adequate concept of the salient gross and microscopic features and the means for diagnosis of the various tumors". The book is written with definite objectives. To "refresh" the knowledge of physician and surgeons and enable them "to glean the essentials without having to sift them out of a mass of historical and theoretical data, academic discussion, clinical discussion and other such material that is regularly found in the few books that deal with the subject of new growths". The author ends the preface by the sentence "This will necessitate a personal, arbitrary and didactic approach which, it is hoped, will be understood and condoned by the reader".

An aggressive attempt has been made to realize the ambitious objectives, which are developed to a degree sufficient to warrant recommendation of the book for the uses indicated by the author.

W B V

Manual of Pharmacology and Its Applications to Therapeutics and Toxicology, A 7th edition. By TORALD SOLLMANN. 1132 pp. \$11.50. W B Saunders Company, Philadelphia, Pennsylvania, 1948.

The seventh edition of Sollmann's "A Manual of Pharmacology" brings a thorough revision of this important text. The far reaching developments of the past decade have called into being new sections on such topics as antibiotic substances, antithyroid drugs, antimalarials, the sulfonamide compounds, and the antihistamine agents. This volume represents a most exhaustive and detailed compilation of the multitudinous details of drug action, well organized, brought up-to-date, presented with discrimination and well documented with references to the original literature. Sollmann's *Manual* represents the most comprehensive reference volume to have at hand when confronted with a specific problem regarding the action of a drug.

M R

Modern Clinical Psychiatry 3rd edition. By ARTHUR P. NOYES. 525 pp. \$6.00. W B Saunders Company, Philadelphia, Pennsylvania, 1948.

This latest edition of Dr. Noyes' widely read textbook contains three new chapters on psychotherapy, shock and other physical therapies and child psychiatry. In discussing psychotherapeutic methods the author stresses the psychobiological approach and presents a rather clear account of what is meant by it. Psychoanalytic methods are adequately described and should acquaint the reader not only with their aims but also with some technical aspects of procedure. Shock therapies are discussed in some detail, however, the author seems fully aware of their limitations, pointing out the importance of adequate psychotherapy being available for patients receiving such treatment. The most useful feature of this book is its dynamic orientation and the author's ability to present dynamic psychiatry in a style which can be easily understood by the student. The experienced psychiatrist

will find it stimulating reading and should be able to make ready use of the very excellent bibliography at the end of each chapter

E A

Noah Webster Letters on Yellow Fever Addressed to Dr William Currie 110 pp \$2 00 *The Johns Hopkins Press, Baltimore, Maryland, 1947*

Eighteenth century America was peculiarly fortunate in its possession of many men whose breadth of interests perplexes the twentieth century specialist. Noah Webster was typical of his time. Not only was he a lexicographer and the author of the American Spelling Book, which ran to 60,000,000 copies, but he was, variously, a lawyer, newspaper editor, statistician, climatologist, economist, and epidemiologist. This small volume of *Letters on Yellow Fever* summarizes some of Webster's epidemiologic views. A contagious disease, he wrote, is one which is communicated from person to person either by contact or near approach. It must be distinguished from infectious disease, which requires for its propagation crowding, hot weather, close rooms, and so forth, all of which generate an epidemic constitution to the atmosphere. Yellow fever belongs to this latter group of diseases. It is useless to quarantine ships from the Indies, since yellow fever is not imported but arises locally as the result of an epidemic constitution to the atmosphere. Moreover, wrote this Federalist, the view that yellow fever is imported is dangerous to our growing commerce. The proper protection against yellow fever is careful planning of our cities to combine the best features of country and city.

These interesting letters are preceded by an entertaining introduction by Benjamin Spector

O D R

Practical Clinical Psychiatry By EDWARD A. STRECKER, FRANKLIN G. EBAUGH AND JACK R. EWALT. 6th Edition. Illus. 476 pp. \$5 00. *The Blakiston Company, Philadelphia, Pennsylvania, 1947*

Although the 6th edition of this popular text has several new chapters, such as those on pathologic drinking and psychosomatic medicine, the general orientation of its contents is essentially unaltered. Written primarily for the medical student and following psychobiological principles, it should prove useful to the beginner to become acquainted with fundamental aspects of psychiatric examination, classification, and, to a lesser degree, with those of psychiatric treatment.

The book's greatest asset is its good organization, and its chief liability is a relative lack of dynamic orientation, particularly as regards the psychoses. The chapter on "Psychosomatic Medicine" is very likely to confuse the reader, being ill-defined and creating the impression that physiological expressions of anxiety as such constitute "psychosomatic" disease.

The chapter on toxic psychosis is very complete and well presented. The problem of alcoholism receives considerable attention in this chapter and also in a separate section on "Pathologic Drinking."

Dr Kanner's discussion of childhood problems is essentially identical with the corresponding chapter of the preceding editions.

In addition to its value to the medical student, the text is likely to serve as a ready reference for the busy practitioner who, as a result of its uncomplicated presentation of clinical psychiatry, should have little difficulty in grasping its contents

E A

Signs and Symptoms Their Clinical Interpretation Edited by CYRIL MITCHELL MACBRYDE Twenty Contributors Illustrated 439 pages \$12 00 J B Lippincott Company, Philadelphia, Pennsylvania, 1947

This is a valuable book for practitioners, medical students and specialists alike. The subject matter is dealt with in monographic form. Twenty contributors of eminence have written upon pain, headache, sore tongue and sore mouth, thoracic pain, abdominal pain, backache and back pain, joint pain, pain in the extremities, fever, disturbances of consciousness and muscle movement, fainting, dyspnoea, cyanosis, dehydration, edema, palpitation and tachycardia, cough, hemoptysis, obesity, weight loss and undernutrition, anorexia, nausea and vomiting, constipation and diarrhoea, hematemesis and melena, jaundice, itching, nervousness and fatigue.

The authors approach diagnosis from an analysis of the account the patient gives of his symptoms and the signs the examiner finds. There are sound discussions of the fundamental pathologic physiology responsible for signs and symptoms. The discussions indicate clearly the lines of special study by clinical and laboratory techniques which will be likely to enable the investigator to arrive at precise diagnoses.

The book is well illustrated both in black and white and in color. There are numerous charts and tables, and the bibliographies, for the most part, are well chosen and complete.

The chapter on headache by Harold G. Wolff is unusually well done. The mechanisms responsible for headaches of all sorts are described. Various factors which enter into the production of clinical pictures of which headache is the chief feature are discussed thoroughly. Anyone not entirely familiar with the problems of head pain will have a very clear concept of the subject after reading this chapter.

The book is enthusiastically recommended

B M B

Submicroscopic Morphology of Protoplasm and its Derivatives By A. FREY-WYSSLING Illus. 255 pp \$6 00 Elsevier Publishing Company, Inc., New York, New York, 1948

This first English edition is really a second edition of "Submikroskopische Morphologie des Protoplasmas und seiner Derivate", published in 1938. At that time it was something of a classic summary of the indirect experimental data indicating a definite ordered structure to material below the microscopic level of vision. Since then a great deal more data, such as that furnished by x-ray patterns, anisotropy of swelling, and polarization microscope techniques have been applied to the study of fine structure. A good summary of this is presented. Data yielded by

the direct method of electron microscopy are presented briefly in the text. It is pointed out that this method cannot be the sole means of asking pertinent questions. New facts or artifacts seen by the electron microscope must be evaluated in relation to all of the other indirect data.

It is too bad, in the light of this, that the few pictures presenting the case for study of submicroscopic structure by electron microscopy are so poor. Perhaps this reflects the lack of availability of such data to European authors during the war, and it is to be expected that this will be corrected in a future edition of this very worthwhile text.

F B B

Textbook of Surgery for Nurses By EDWARD S. STAFFORD AND DORIS DILLER
Illus. 577 pp. \$3.25 W. B. Saunders Company, Philadelphia, Pennsylvania, 1947

This is an excellent text. The style is simple and informal and the subject matter a tribute to the dignity of the nursing profession. The approach is based on the conviction that a nurse will give better care to patients if she understands the general pathology of a disease and the aims and technics of the surgeon in treating them. Each important condition is therefore introduced with appropriate background material, and then the matters pertaining more strictly to ward nursing are brought in and their rational basis made clear. Two-thirds of the book is on surgery. The remaining portion is devoted to the special fields of surgery: neurosurgery, ophthalmology, otolaryngology, genito-urinary, and gynecological surgery. There is an abundance of common sense throughout the book based on the wide experience of the two authors. Although much of the material is written so straightforwardly that it would be interesting reading for a layman, the book could be recommended strongly to third and fourth year medical students who are about to begin their ward apprenticeships. It is expected to have a wide field of usefulness in the training of nurses. The illustrations are numerous and are diagrammatic in type.

W E G

Treatment in General Practice 6th edition By HARRY BECKMAN 1129 pp
\$11.50 W. B. Saunders Company, Philadelphia, Pennsylvania, 1948

This new edition of an old favorite textbook lives up in every way to the high standards of the previous editions. The general pattern of preceding volumes is closely followed, but a large amount of revision shows in almost every topic. Recent advances are well covered. The bibliography is large and well chosen, as well as up to the minute. The style is as engaging as ever, written in a personal way, amusing and even a trifle saucy, but always quickly moving and rarely bogging down in argument or disagreements. The reader may not agree with every detail, but the over-all picture is clear and well presented. This edition is highly recommended as a textbook or reference source.

J A L, Jr

BOOKS RECEIVED FOR REVIEW

- Arterial Hypertension* By DAVID AYMAN Illus 89 pp \$2 50 Oxford University Press, New York, New York, 1948
- Chemical Laboratory Methods and Diagnosis* 4th edition, 3 vols By R B H GRADWOHL Illus 3148 pp \$40 00 The C V Mosby Company, St Louis, Missouri, 1948
- Clinical Picture of Thyrotoxicosis, The* By PETER MCEWAN Illus 127 pp 15s net Oliver and Boyd, London, 1948
- Communicable Diseases*, 2nd edition By FRANKLIN H TOP Illus 992 pp \$9 50 The C V Mosby Company, St Louis, Missouri, 1948
- Conference on Metabolic Aspects of Convalescence* Edited by EDWARD C REIFENSTEIN, JR Illus 163 pp \$2 25 Josiah Macy, Jr Foundation, New York, New York, 1948
- Economic Man in Relation to his Natural Environment*, 2 vols By C REINHOLD NOYES 1443 pp \$15 00 Columbia University Press, New York, New York, 1948
- History of the Heart and the Circulation, A* By FREDERICK A WILLIUS and THOMAS J DRY Illus 456 pp \$8 00 W B Saunders Company, Philadelphia, Pennsylvania, 1948
- Human Physiology*, 3rd edition By F R WINTON and L E BAYLISS Illus 592 pp \$7 00 The Blakiston Company, Philadelphia, Pennsylvania, 1948
- Identification of Tumors* By N CHANDLER FOOT Illus 397 pp \$6 00 J B Lippincott Company, Philadelphia, Pennsylvania, 1948
- Intracranial Tumors*, 2d edition By PERCIVAL BAILEY Illus 478 pp \$10 50 Charles C Thomas, Springfield, Illinois, 1948
- Mechanism of Abdominal Pain, The* By V J KINSELLA Illus 230 pp 32s 6d Angus & Robertson, Ltd, Sydney, Australia, 1948
- Medical Research in War* Report of the Medical Research Council for the years 1939-45 455 pp 7s 6d net H M Stationery Office, London, W 1, England, 1948
- Modern Clinical Psychiatry*, 3rd edition By ARTHUR P NOYES 525 pp \$6 00 W B Saunders Company, Philadelphia, Pennsylvania, 1948
- Practical Bacteriology, Hematology, and Parasitology*, 10th edition By E R STITT, PAUL W CLOUGH, SARA E BRANHAM, and contributors Illus 991 pp \$10 00 The Blakiston Company, Philadelphia, Pennsylvania, 1948
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- Treatment of Heart Disease* By WILLIAM A BRAMS Illus 195 pp \$3 50 W B Saunders Company, Philadelphia, Pennsylvania, 1948
- War, Politics, and Insanity* By C S BLUEMEL 121 pp \$2 00 The World Press, Inc, Denver, Colorado, 1948

SYMPOSIUM ON THE PHYSIOLOGY OF ACETYLCHOLINE

I THE RÔLE OF ACETYLCHOLINE IN CONDUCTION*

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INTRODUCTION

For more than a century neurophysiology was limited to the study of the electrical signs of activity, but the necessity for an understanding of the underlying chemical mechanism was well recognized by leading neurophysiologists. The small electric currents which propagate the nerve impulse cannot derive the energy from the stimulus itself, the energy must be supplied locally by a "propagated disturbance," as Keith Lucas pointed out (1). Experimental confirmation of this view was obtained in 1926, when A. V. Hill succeeded in measuring the heat production and O. Meyerhof the extra oxygen uptake connected with nerve activity. The difficulty of studying the chemical reactions directly responsible for the propagation of the impulse is easily understood if we keep in mind two essential features of conduction: (i) the high speed, and (ii), the small amount of energy required. According to A. V. Hill, the initial heat production in a frog sciatic per gram per impulse is, at room temperature, of the order of magnitude of 1 to 2 ergs or 10^{-8} gram calories. The energy released by the oxidation of one gram of sugar is equivalent to the initial heat of several hundreds of billions of impulses per gram nerve. The amount of any chemical substance metabolized in this primary event is most likely of an order of magnitude far below the range of our best micromethods. Since, moreover, these chemical reactions take place in a period of time shorter than one thousandth of a second, probably close to one ten-thousandth of a second, it is obvious that direct chemical measurements of the active substances are at present impossible.

However, stimulated by the pioneer work of O. Meyerhof, much

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valuable information about the chemical mechanism of another cellular function, that of muscular contraction, has been obtained during the last two decades in a different way, viz, by the study of the enzyme systems which catalyze the substances involved. It is true that enzyme activities measured *in vitro* indicate only potential reaction rates. Such studies can supply, therefore, only indirect information. But if we succeed in correlating the enzyme activities in the living cell with function recorded by physical methods, the result may be as conclusive and as direct as that which would be obtained by a similar correlation of the substrate metabolized by the enzyme. This kind of approach appeared particularly promising for the study of the chemical mechanism of conduction in view of the high rate and the small quantities of substrate metabolized.

During the last fifty years, two chemical compounds were specifically connected with nerve activity: adrenaline and acetylcholine. Early in this century, R. T. Elliot (2) suggested that adrenaline might be released from sympathetic nerve endings and act as a chemical mediator of the impulse transmitting it from the nerve endings to the effector organ. This idea made a profound impression on many physiologists. Especially after the observations of Loewi and Dale, that acetylcholine may appear under certain conditions in the perfusion fluid following the stimulation of certain nerves, the theory of neurohumoral or chemical transmission found many supporters. The theory stimulated a great number of investigations and many interesting and valuable observations resulted from these studies. There were, however, many difficulties and contradictions, and neurophysiologists became increasingly opposed to the idea (Erlanger (3), Fulton (4)).

During the last twelve years, the enzymes connected with the formation and hydrolysis of acetylcholine were studied and correlated in many ways with the electrical manifestations. These investigations have shown that the hypothesis of chemical transmission as proposed originally has to be modified. The release and the removal of acetylcholine are intracellular processes occurring in the neuronal surface and are a necessary link in the chain of reactions which generate the small electric currents conducting the impulse. The facts on which the assumption of a rôle of acetylcholine in the conduction of the nerve impulse has been built have been reviewed (Nachmansohn

5-7) In the first part of the lecture, the most important results of these investigations may be briefly summarized. It may be stressed, however, that this paper is limited to a few aspects pertinent to the rôle of acetylcholine and that other developments important for the understanding of conduction cannot be discussed.

I BASIC FACTS IN FAVOR OF A RÔLE OF ACETYLCHOLINE IN CONDUCTION

A *Cholinesterase*

Five essential features of physiological significance emerged from the studies on cholinesterase: (i) the ubiquity of the enzyme in all conductive mechanisms (nerve and muscle) throughout the whole animal kingdom. The enzyme is present in the *Tubularia*, a hydrozoan coelenterate, which possesses the lowest form of neuromuscular tissue comparable to that of higher groups of animals (8). (ii) the exclusive localization of this enzyme in the neuronal surface where the bioelectric phenomena occur (9). (iii) the extremely high speed at which the hydrolysis of acetylcholine by cholinesterase may occur. The "turn-over number" of this enzyme is about twenty million per minute. One molecule of enzyme may split one molecule of acetylcholine in about 3 to 4 microseconds (10). This speed is perhaps the most outstanding feature of the enzyme system since it is a prerequisite for any assumption connecting a chemical reaction directly with electrical manifestations. No other chemical reaction known to be connected with nerve activity has a comparable speed. (iv) a significantly high concentration of the enzyme in all nerve tissue. From the data obtained with the giant axon of Squid, e.g., it was calculated that one square mm of neuronal surface is capable of splitting 1×10^9 molecules of acetylcholine in a thousandth of a second. (v) the specificity of the enzyme in nerve and muscle tissue for acetylcholine. The enzyme is distinctly different from the esterases of other tissues, although the specificity is relative and not absolute, as could be expected in the case of an esterase (11).

B *Correlation between cholinesterase and nerve activity*

All these features of the enzyme are suggestive and valuable as indirect support for the assumption that the activity of this enzyme is important for conduction. For demonstrating directly the rôle of

cholinesterase during activity, it was necessary to correlate the enzyme activity with the electrical manifestations. This has been achieved in several ways

1) *Direct proportionality between voltage and cholinesterase activity in electric tissue* The first such correlation was established in experiments on electric fish. The electric tissue has phylogenetically evolved from muscle. It is generally agreed that the electric discharge in this tissue is fundamentally identical with the action potential in nerve and muscle. The high voltage is due to the arrangement of the elements in series. Each physiological unit, the electric plate, produces about 0.1 volt which is the same order of magnitude as observed in other nerves and muscles. The electric plates are homologous to motor end-plates. The contractile elements have disappeared in the strong electric organs but they exist as rudiments in weak electric organs. The remarkable structure of the electrolemma surrounding the nerve endings in the electric organ, has revealed interesting similarities with the post-synaptic membrane at the motor end-plate, as was recently shown by Couteaux (12). The electric discharge in this fish is more exactly homologous to the end-plate potential developed in the post-synaptic membrane of the neuromuscular junction.

Great variations in the number of electric plates per cm are found in the electric organ of the *Electrophorus electricus*, the South American electric eel. The frequency varies considerably with the size of the specimen and decreases from the head to the caudal end of the organ in each specimen. Since each plate develops, as mentioned above, about the same voltage, the variations of the voltage per cm are considerable. A close parallelism was found between the number of electric plates, the voltage per cm, and the concentration of cholinesterase (13). Studying this relationship on a large number of specimens covering a range from 0.5 volt to 22 volts per cm, it was found that if the voltage per cm is plotted against the cholinesterase concentration, the resulting line calculated by the method of least squares goes virtually through zero, which indicates a direct proportionality between voltage per cm and cholinesterase activity (14). This finding is in striking contrast to the distribution of other enzymes and compounds like adenosinetriphosphatase, respiratory and glycolytic enzymes, phosphorylated substances, etc., which do not show any

significant variation in concentration in relation to the voltage developed. The direct proportionality between physical and chemical processes suggests an interdependence of the two events.

2) *The discovery of choline acetylase*. The electric organ is an unusually favorable material for correlating electrical and chemical events. In view of the small energy involved in conduction, the chemical reactions in ordinary nerves are not within easy range of measurement, whereas in the electric tissue it is possible with the available methods to correlate the electrical and the chemical energy released during activity. It was found that the energy released by the breakdown of phosphocreatine per gram per impulse is adequate to account for the total electrical energy (15). It has been established by the work of Otto Meyerhof and his school that the phosphocreatine is only a storehouse for energy-rich phosphate bonds and that the breakdown of adenosinetriphosphate precedes that of phosphocreatine. It is believed today, on the basis of the work of Engelhardt and Ljubimova, Needham, Szent-Gyorgyi, and their associates, that adenosinetriphosphate may react directly with the muscle protein and that its breakdown may be the primary chemical reaction during muscular contraction. It was safe to assume therefore that the breakdown of adenosinetriphosphate occurs during nerve activity as in muscle, before that of phosphocreatine. But, it appeared unlikely for many reasons that the adenosinetriphosphate breakdown is the primary reaction connected with conduction, as recently proposed (16), because of the marked differences between conduction and contraction. Among other obstacles, the greatest difficulty is the time factor. There is no evidence that adenosinetriphosphate breakdown may occur at the speed required for the primary event in conduction. The turnover number of adenosinetriphosphatase is 8,000 per min., as compared with 20,000,000 for cholinesterase. On the basis of the available evidence, it appeared more likely to assume that the release and removal of acetylcholine are the primary reactions connected with the changes of the protein or lipoprotein in the active surface membrane. The breakdown of adenosinetriphosphate was assumed to be the primary recovery process supplying the energy for the resynthesis of the acetylcholine hydrolyzed during the passage of the impulse. If this is correct, the energy of adenosinetriphosphate should be used for

the resynthesis of acetylcholine (17) This hypothesis was tested in the following way A brain extract was prepared, to the cell-free solution obtained, acetate, choline, and adenosinetriphosphate were added Other additions were eserine for protecting the acetylcholine formed against the action of cholinesterase, and fluoride for preventing the too rapid breakdown of adenosinetriphosphate, since fluoride, according to Ochoa (18), inhibits the activity of adenosinetriphosphatase but not that of the phosphorus-transferring enzyme It was found that the cell-free solution contains an enzyme which forms acetylcholine at a high rate under strict anaerobic conditions, using the energy of adenosinetriphosphate (19) The enzyme was called choline acetylase The discovery of this enzyme is consistent with the hypothesis formulated above, viz, that the breakdown of acetylcholine precedes the breakdown of adenosinetriphosphate during the discharge of the impulse and that the energy of the adenosinetriphosphate is used for the resynthesis of acetylcholine At that period, in 1943, when this hypothesis was proposed and tested, no other chemical reaction outside the glycolytic cycle was yet known to use the energy of adenosinetriphosphate It was the first demonstration that energy-rich phosphate bonds may be used for anaerobic acetylation

The whole enzyme system is rather complex but has been completely reconstructed *in vitro* (20-24) The most active preparations obtained from 150 mg of acetylcholine per gram protein per hour The biochemical aspect need not be discussed here But two facts of physiological interest may be mentioned, which emerged from the studies with this enzyme system First, the enzyme is present in all nerve fibers and also in muscle (25) The absence of acetylcholine in sensory nerves was considered for a long time, an obstacle for the assumption that this ester is essential for conduction Evidence for the presence of choline acetylase in sensory nerves removes this objection since the presence of both choline acetylase and cholinesterase indicates that acetylcholine is metabolized there as well as in motor nerves This is confirmed by the observations discussed later, that inhibition of cholinesterase blocks conduction in sensory as well as in all other types of nerves It is true that the rate of synthesis in sensory seems to be slower than in motor nerves In the optic nerve of the rabbit, 15 to 20 μg of acetylcholine were found to be formed per gram nerve per

hour as compared with 80 to 110 μg in the motor nerve, although this rate is certainly not the maximum considering the technical difficulties (23). But quantitative differences of chemical reactions are to be expected in view of the many variations of the structural and electrical properties, the cholinesterase activity varies also considerably, not only in different types of nerves, but even in the same type of nerve in different species and groups of animals.

Another point of physiological interest is the behavior of choline acetylase during the degeneration of the nerve fiber. It was claimed that the ability of nerves to form acetylcholine disappeared before failure of conduction (Feldberg (26)). The whole concept of a rôle of acetylcholine in conduction was therefore assailed (27). These observations, however, were based on inadequate techniques. Actually, more than one third of the choline acetylase activity is still present at the time when conduction disappears (22). Thus, the formation of acetylcholine considerably outlasts the ability for conduction.

3) *Effect of anticholinesterases on conduction* Obviously, if the rapid removal of the acetylcholine released during the passage of the impulse is necessary for conduction, interference with this mechanism should block conduction. This prediction was tested by the use of anticholinesterases. It could be shown that if nerves are exposed to anticholinesterases, like eserine and strychnine, the action potential is abolished (28). Since the enzyme inhibitor complex is easily reversible in the case of these compounds, washing of the nerves should restore conduction. This has been demonstrated with a variety of nerves. After abolition of the action potential by exposure of a nerve to eserine, subsequent washing evoked complete recovery. This cycle could be repeated several times on the same nerve.

II THE MECHANISM OF DI-ISOPROPYL FLUOROPHOSPHATE (DFP) ACTION

It was at this stage of development that investigations with a new anticholinesterase, di-isopropyl fluorophosphate (DFP), seemed to contradict the assumption of the necessity of cholinesterase for conduction. These observations were presented at a symposium held at the New York Academy of Sciences early in 1946 (29). The discovery of this compound posed two distinct problems. First, whether the

high toxicity could be attributed exclusively to the reaction with cholinesterase. Second, whether it was possible, by exposure of nerves to this compound, to destroy cholinesterase irreversibly without impairing conduction. As pointed out by Dixon and Needham (30), DFP is one of the most powerful and most specific enzyme inhibitors. Among 20 enzyme systems tested, only the esterases were affected. There is growing evidence that compounds which are extremely potent, i.e., act in very low concentrations, produce their effect by interference with enzymes. It appeared then possible to assume that the high toxicity of DFP has to be attributed to its action on cholinesterase. Many difficulties and apparent contradictions, however, had to be overcome until a clear picture was obtained, and I would like to devote the second part of the lecture to the rather dramatic development of the last two years in connection with the investigations of the mechanism of DFP action.

A Reversibility of cholinesterase inhibition by DFP Early reports seemed to indicate that the irreversible inactivation of cholinesterase by DFP is instantaneous. Since abolition of conduction in nerves exposed to DFP can be reversed, a completely irreversible immediate destruction of cholinesterase appeared incompatible with the assumption that the effect on conduction is due to the inactivation of cholinesterase. It was suggested that the conduction in nerves exposed to DFP is blocked by a general toxic effect of this compound.

It has been shown, however, that the irreversible inactivation of cholinesterase by DFP is not an instantaneous process but progresses rather slowly. The rate of irreversible inactivation depends on a number of controllable factors like temperature, concentration of inhibitor, and others (31). It could be demonstrated that, at low temperatures, the process may be partly reversed even after 2 to 3 hours incubation of the enzyme with DFP. The higher the temperature, the greater the rate of the irreversible process, the Q_{10} being about 2. The concentration of the inhibitor is an important factor. For a given time of incubation, the range of the concentration at which reversibility may be demonstrated is rather small. Fig. 1 illustrates the dependence of the reversibility of the enzyme inhibition upon the DFP concentration.

B Parallelism between abolition of conduction and inactivation of

cholinesterase This peculiar feature of cholinesterase inhibition by DFP, viz, its slowly progressive irreversibility, made it possible to establish conclusively the necessity of cholinesterase for conduction. A most striking parallelism has been demonstrated between the progressive irreversible inactivation of cholinesterase activity and the

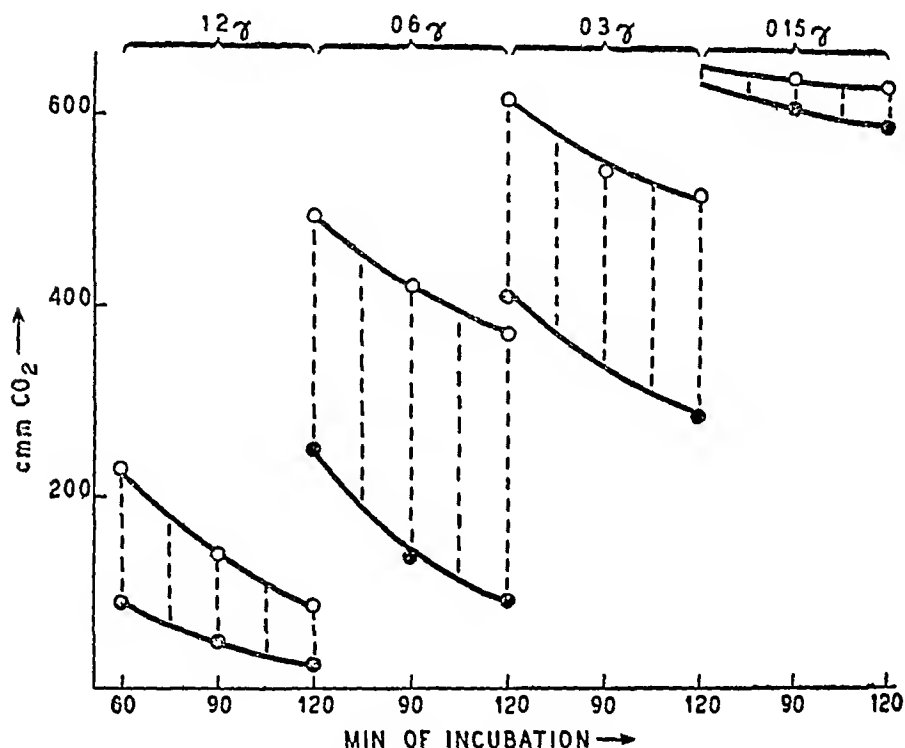


FIG 1 REVERSIBILITY OF CHOLINESTERASE INHIBITION ON EXPOSURE TO VARYING CONCENTRATIONS OF DFP FOR 60-120 MIN AT 7°C, TESTED BY DILUTION METHOD ELECTRIC TISSUE ESTERASE
●—●—●, undiluted ○—○—○, diluted after exposure

progressive irreversible abolition of conduction. The giant fibers which run through the abdominal chain of the lobster were selected for these experiments, since the cholinesterase activity here is high and the preparation therefore favorable for correlating electrical and chemical processes (32).

In these experiments the nerves were exposed to DFP for varying

periods of time and then washed with seawater. The longer the period of exposure, and the less complete the recovery of the action potential, the smaller was the activity of cholinesterase remaining in the nerves.

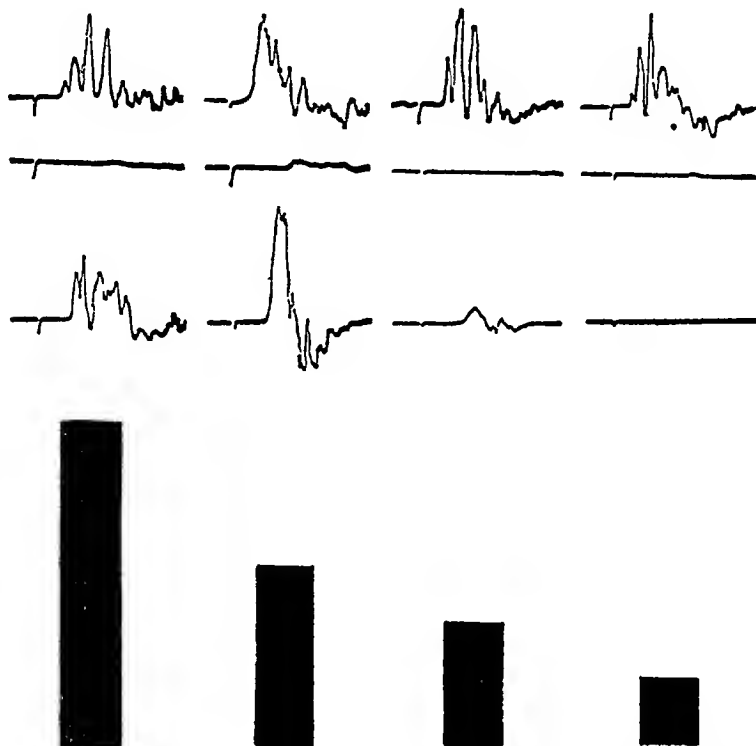


FIG 2 PARALLELISM BETWEEN THE PROGRESSIVE IRREVERSIBLE ABOLITION OF CONDUCTION AND THE PROGRESSIVE IRREVERSIBLE INACTIVATION OF CHOLINESTERASE IN LOBSTER NERVES EXPOSED TO DFP

Four nerve preparations shown in the 4 columns were kept in DFP (0.013M) for 30, 60, 90, and 120 min respectively and then washed in sea water. The top line of each column shows the action potential in the untreated nerves. The second line shows the abolition of the response by DFP. The third line shows the degree of recovery of the action potential after prolonged washing of the nerve. The cholinesterase activity still present is shown in the vertical bars of the fourth line. The CO_2 output is 233, 129, 88.5 and 50 cmm per 100 mg per hour as compared with about 2000 cmm CO_2 in the normal preparation.

When conduction was irreversibly abolished, the activity of the enzyme was 5 percent or less of the initial (Fig 2). The same parallelism has been obtained in experiments with other nerves, especially also with

the giant axon of Squid, a single fiber preparation. In all instances where conduction could be restored, cholinesterase activity was still present (33).

The parallelism between cholinesterase activity and conduction has been established, not only as a function of time but also as a function of temperature. It takes about $1\frac{1}{2}$ to 2 hours to abolish conduction at room temperature, and about 3 to 4 hours at 5 to 10°C, as could be expected on the basis of the rate of the irreversible inactivation of cholinesterase *in vitro*. According to these data, it should take only 20 to 30 min. for obtaining this effect at 37°C. This was tested with the superior cervical sympathetic of the cat. If the nerves were exposed for a few minutes, and the action potential abolished, washing with Ringer's solution could restore conduction. But, in about 20 minutes, the process became irreversible (Fig. 3). Here again, cholinesterase was found as long as the action potential reappeared. No cholinesterase was found if conduction was irreversibly abolished after exposure to DFP (34).

The same effect could be demonstrated with all types of nerves, myelinated and unmyelinated, vertebrate and invertebrate, motor and sensory nerves, adrenergic as well as cholinergic nerves (8). Finally, it was also shown for striated muscle (35). As mentioned above, cholinesterase is present in all conductive tissue (nerve and muscle) throughout the whole animal kingdom. Since in all types of nerves and muscle inactivation of cholinesterase leads to abolition of conduction, the observations support the generality of the rôle of cholinesterase in all conductive mechanisms.

C Impossibility of dissociating cholinesterase activity and conduction

It is impossible under any conditions to dissociate conduction and cholinesterase activity. It is true that it is sometimes difficult to demonstrate cholinesterase activity in nerves which were exposed to DFP. This difficulty, however, is due to the fact that normally the enzyme is present in excess, which amounts in some nerves to about 10 to 12 times the concentration necessary for normal function. In other words, 90 to 92 percent of the cholinesterase present in a nerve may be inactivated without impairing conduction, but the remaining 8 to 10 percent is essential. An excess of 10 times is not unusual on the basis of our experience with other enzyme systems. An excess of



FIG 3 *A* Reversible effect on the superior cervical sympathetic nerve of the cat exposed to DFP for 2 min. Records from top to bottom: normal, after 2 min exposure, recovery. *B* Irreversible effect on the superior cervical sympathetic exposed to DFP for additional 20 min after abolition of the action potential. Records from top to bottom: normal, 3 min exposure, after 120 min in Ringer's solution. 1000 c.p.s.

50 or 100 times may be found. But whereas the normal cholinesterase activity in most nerves is easily determinable, the remaining activity

after inactivation of the excess is sometimes small in absolute figures and its determination requires therefore special precaution. In the experiments of Gilman and his associates (36), on the bullfrog sciatic nerve, the manometric technique used was inadequate for detecting the remaining cholinesterase activity due to several adverse factors (33). An activity equivalent to 7 to 10 percent of the initial activity can, however, be demonstrated in the following way: the homogenized nerves are added to a solution of acetylcholine, the disappearance of the ester is measured by the determination with the frog's rectus method.

Boyarski, Tobias, and Gerard (37) exposed the frog sciatic nerve for several hours to a concentration of DFP just below that which affects the action potential. Under these conditions, they were unable to detect cholinesterase although they used for the determination of cholinesterase activity the method just described. Unfortunately, in their determinations, the ratio of enzyme to substrate was inadequate. Under exactly the same experimental conditions, but taking for the determinations more nerve and less substrate, it has been shown that the nerve contains a cholinesterase concentration capable of splitting 400 to 500 μg of acetylcholine per gram per hour (38). For the evaluation of this activity, it may be useful to recall the small initial heat in these nerves. At 23°C at a rate of 300 to 400 impulses per second, the initial heat per gram per impulse is about $2-3 \times 10^{-8}$ gcal. If acetylcholine acts as a trigger in the chain of reactions associated with the flow of current, the hydrolysis of the ester will account for only a fraction of the initial heat. Let us assume, for instance, for 20 percent. This would be equivalent to $4-6 \times 10^{-9}$ gcal. On the basis of 2,000 gcal released per mole of acetylcholine hydrolyzed, this would amount to 0.0003 to 0.0004 μg of acetylcholine split per gram per impulse. The ability to split 400-500 μg of acetylcholine per gram per hour would then account for one to two million impulses during this period (39).

D. Coincidence of cholinesterase inactivation with death. If cholinesterase activity is essential for conduction, survival of an animal should be impossible in the absence of cholinesterase. However, observations were reported that animals may survive in complete absence of the enzyme in the brain (40). The question has been re-examined. It was found that absence of cholinesterase in the brain

always coincides with death (39) If a threshold dose is injected in which some animals survive, others die, in the surviving animals cholinesterase was always present without a single exception, whereas in the others the enzyme had disappeared The average concentration found in the surviving animals was about 25 percent of the initial value, varying between 10 and 50 percent Other enzymes were not affected

In this connection the recent observations of Freedman and Himwich (41) are important These investigators found that during recovery from DFP poisoning the signs of toxicity diminish as the brain cholinesterase rises Another significant finding in this connection is that of Jones, Meyer and Karel (42) The acute toxicity of a great number of organic phosphorus compounds tested was found to be a function of their potency as cholinesterase inhibitors

E The concentration of DFP required for abolition of conduction
One of the main objections to the assumption that DFP abolishes conduction by inactivation of cholinesterase is the concentration used DFP inactivates cholinesterase *in vitro* in a concentration of a few μg per cc, whereas the concentrations used to abolish conduction are 1 to 2 mg of DFP per cc This difference was considered by Feldberg and Mendel as evidence that the abolition of conduction by DFP is due to a general toxic effect, rather than to the inactivation of cholinesterase (Discussion at the International Congress of Physiology at Oxford in 1947) The objection does not take into account one of the most fundamental factors on which the action of a compound applied to a living cell depends, namely, the structure The concentration outside the cell is irrelevant The decisive factor is the concentration at the site of action Nerve fibers are surrounded by a myelin sheath and other membranes, which may form either a complete barrier for the penetration of some compounds into the interior or slow down the rate of penetration Using the giant axon of Squid, we have tested the concentration of DFP in the interior of the cell at the time when the action potential disappears (38) This concentration was found to be of the order of magnitude of 1 μg per cc, or one per mill of the outside concentration Sometimes it was lower, and in one case it was about 5 per mill The inside concentration at the time when conduction disappears was about the same, whether 400 μg of DFP per cc or 1 mg

per cc were used outside. These observations show that the concentration inside the cell may be only about one-thousandth of the outside concentration at the critical moment. They answer satisfactorily the question why the outside concentration has to be so much higher than that used to abolish cholinesterase activity if the enzyme is in solution.

F Kinetic aspects of cholinesterase inhibition by DFP Even *in vitro*, the effectiveness of DFP depends on a great number of factors. The importance of temperature and concentration of inhibitor for determining the rate of the irreversible inactivation of the enzyme by DFP were previously mentioned. There are, however, other factors.

The inhibition of cholinesterase by DFP occurs on a mole-to-mole basis (43). This could be demonstrated in experiments on virtually pure cholinesterase prepared from electric tissue in which 1 mg of protein splits 60 gm of acetylcholine per hour. The enzyme was incubated with varying concentrations of DFP for 150 min at 10°C. The solution was then diluted several thousand times, whereby the action of DFP was interrupted instantaneously and the remaining activity was tested. The amount of enzyme inactivated changes directly proportionally with the concentration. The inhibitory effect depends, however, not only upon the concentration of the inhibitor but also upon that of the enzyme. If pE , the negative log of the molar concentration of the enzyme is plotted against the log of the ratio of inhibitor concentration to enzyme concentration, a straight line is obtained (Fig 4). The excess of inhibitor required to produce 50 percent inhibition in a given time increases rapidly with dilution. In a solution of which 1 cc splits 12 gm of acetylcholine per hour, the excess of molecules of inhibitor over molecules of enzyme is 25. In a solution in which 1 cc splits 1 mg of acetylcholine per hour, which is a concentration of the order of magnitude used generally with the Warburg manometric method, more than 100,000 molecules of inhibitor are necessary for each molecule of enzyme in order to obtain the 50 percent inactivation.

The most important difference between the inhibition of cholinesterase by DFP and that by the alkaloids, eserine and prostigmine, is the easily reversible nature of the reaction with the alkaloids in contrast to the progressive irreversible effect of DFP. But here again, even

in vitro, many other differences may be demonstrated which affect the course of the action. Whereas, e.g., the alkaloids act immediately and the percentage inhibition remains unchanged for a considerable period of time, the inhibitory effect of DFP increases constantly. Hereby

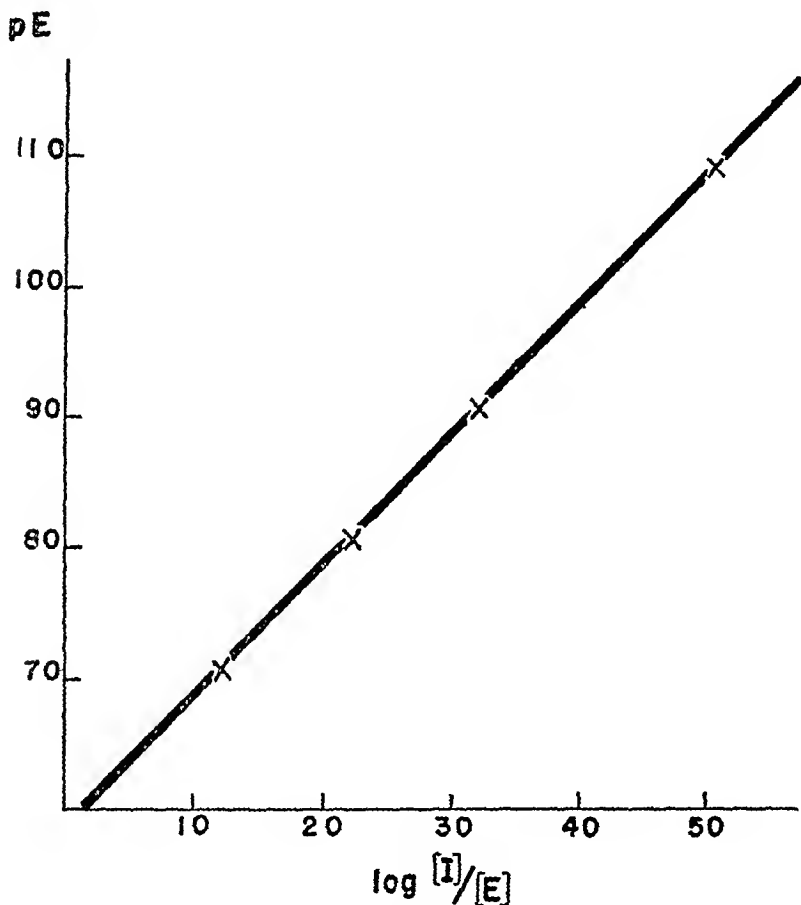


FIG. 4. EXCESS OF DFP REQUIRED FOR VARYING ENZYME CONCENTRATIONS

pE , the negative log of the molar concentration of the enzyme is plotted against the log of the ratio of inhibitor concentration $[I]$ to the enzyme concentration $[E]$

the relationship between the inhibitory effects of the alkaloids, compared to that of DFP, becomes rather complex. But, even if we compare the two inhibitory effects for a given time of incubation, and compare percentage inhibition produced by the two types of inhibitor, we find that in low concentrations the alkaloids have a stronger effect

With increasing concentrations, the effect of DFP becomes stronger and rapidly reaches completion, whereas the inhibition by the alkaloids shows a more asymptotic type of line (Fig 5)

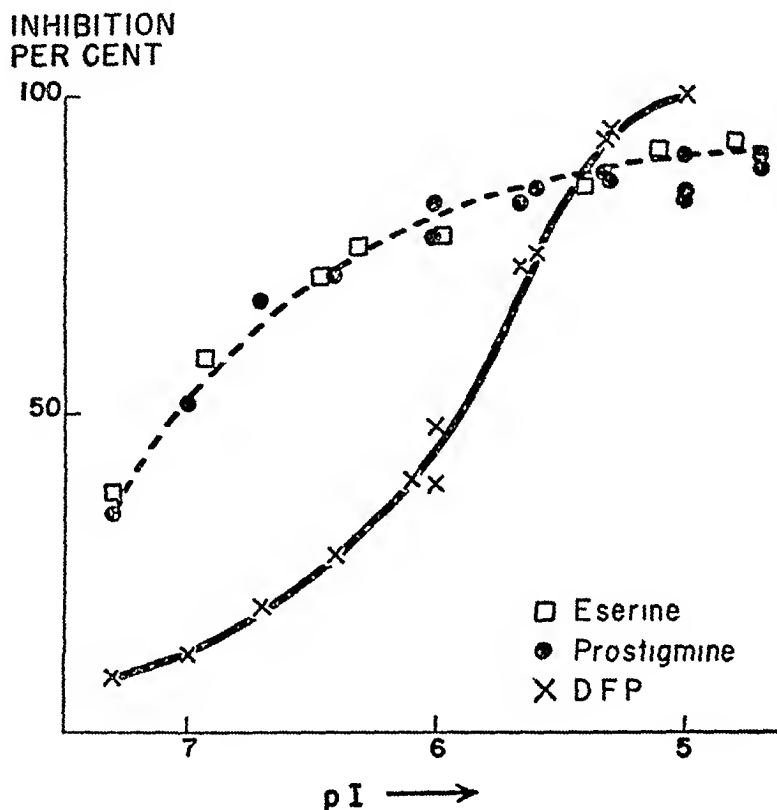


FIG 5 EFFECTIVENESS OF THE INHIBITION OF CHOLINESTERASE BY DFP AND THE ALKALOIDS PROSTIGMINE AND ESERINE AT VARYING INHIBITORY CONCENTRATIONS

Inhibition in per cent plotted against pI , the negative log and the molar concentration of the inhibitors. In the experiments with DFP, the enzyme was incubated for 150 min, with the inhibitor before the determination. No incubation time was used in the experiments with the alkaloids.

The observations described may suffice to demonstrate upon how many factors the effectiveness of the enzyme inhibition *in vitro* depends. Even more factors have an essential rôle in determining the effects on cells and organs *in vivo*, such as circulation, permeability of

different membranes, rate of penetration, etc. Consequently, it is not surprising that the toxic signs due to DFP are variable but this variety of effects must be attributed not to a multitude of chemical reactions but to the many factors which have been demonstrated to influence the course of this single reaction

G Protection by alkaloids against DFP action The finding that DFP acts on cholinesterase on a mole-to-mole basis is of importance in another respect. On the basis of the sedimentation rate in the ultracentrifuge with the preparation which has only one protein component, it was calculated that the molecular weight of cholinesterase is about three million. The molecular weight of DFP is only 180. The stoichiometric relation suggests that the DFP may act on the active group which determines the activity of the enzyme. Goldstein (44) has offered evidence that eserine may act on serum esterase on a mole-to-mole basis. Prostigmine and eserine, of a molecular weight of about 300, act on cholinesterase in concentrations similar to those of DFP. It appeared possible that they act on the same active group. If so, when the enzyme is incubated with prostigmine prior to the incubation with DFP, the active group should be bound by the prostigmine and become inaccessible to the DFP. This assumption was confirmed by the following experiment. Cholinesterase was incubated for 20 min with prostigmine. DFP was then added in exactly the same molar concentration which, without prior incubation with prostigmine, produces about 50 percent irreversible inactivation within 150 min. But with prostigmine added prior to DFP, at the end of the experiment, when the preparation was diluted several thousand times, the total cholinesterase activity was still present. The prostigmine on a mole-to-mole basis had protected the enzyme against the action of DFP. If the molarity of prostigmine used was only one-half of that of DFP, the protecting effect was only about 50 percent. Koelle (45) had already described the protecting effect of eserine against the action of DFP in brain homogenates. The present observations confirm his finding and show in addition the quantitative relationship.

The observation has been used to demonstrate in a new way that the action of DFP on conduction is due primarily to the inactivation of cholinesterase. The experiments were carried out in the following way. The superior cervical sympathetic of the cat was first exposed to eserine in 0.02M concentration until conduction was abolished

This occurs usually within 10 to 20 min. At this moment it had to be assumed that the active group of cholinesterase was associated with eserine and therefore protected against the effect of DFP. DFP was now added to the solution in the same high concentration, 0.02M for about 20–30 min. This time of exposure to the concentration of DFP used is usually sufficient to kill the nerve, i.e., to abolish irreversibly conduction and to inactivate irreversibly all cholinesterase activity. After 20 min the nerve was put back into eserine to permit the DFP to diffuse out of the nerve into the bathing fluid, and after 40 min the nerve was put back into Ringer's solution and washed for 3 hours. Whereas, in the control nerve treated only with DFP for the same time and at the same concentration, conduction was completely abolished and cholinesterase absent, in the eserine treated nerve both conduction and cholinesterase came back (unpublished data). The observations add new support to the assumption that DFP acts exclusively by its reaction on cholinesterase and illustrate how the electrical manifestations may be predicted and controlled on the basis of the chemical reactions observed *in vitro*.

The challenge to the assumption that acetylcholine plays an essential rôle in conduction has thus been met successfully. Having passed this test, the idea emerged stronger than before. The investigations on the mechanism of DFP have not only given us valuable information in the field of general toxicology, but, in addition, have shown that this compound is an extremely valuable tool for the study of some fundamental aspects of nerve activity.

Although the necessity of acetylcholine for conduction has now been conclusively established, the precise function of the ester remains open to discussion. According to the widely accepted membrane theory, proposed early in this century by Bernstein, the nerve is surrounded by a polarized membrane, the positive charge being on the outside and the negative on the inside. During the passage of the impulse, the polarization breaks down and the charge is even reversed, as has been shown by Hodgkin and Huxley (46, 47), and Curtis and Cole (48). The electromotive force between the inside and the outside has most likely to be attributed to the difference of the ion concentrations. Recent observations of Hodgkin and Huxley indicate indeed that, during activity, potassium ions leak out and sodium ions move in. Such ion movement occurs in resting condition, as has been shown by

Rothenberg (49, 50) on the giant axon of Squid with the aid of radioactive isotopes. However, an exchange at a much higher rate will take place during activity. It is known that the resistance falls during the passage of the impulse from 1,000 to 40 ohms per square mm (51). It is a possibility that this change in resistance, i.e., the increased permeability for ions during conduction, is due to the release of acetylcholine and its effect on the proteins or lipoproteins of the active membrane. A compound which may be metabolized in a few microseconds may well account for such a change. Moreover, the observations of Fessard on the electric tissue of *Torpedo Marmorata* have shown the ability of the ester to change the membrane resistance and to generate an action potential (52, 53). The high potency of the ester, indicated by the small amounts which produced the electrogenic effect, becomes a pertinent feature in connection with the high rate of its metabolism.

III AXON AND SYNAPSE

In contrast to the anticholinesterases discussed so far, prostigmine has no effect on conduction in nerve or muscle. Prostigmine is *in vitro* a very strong anticholinesterase. The inhibitory effect on a molar basis is equal to that of eserine, and the kinetics of this inhibition, as mentioned above, are, in all respects tested, identical. Why do we find then, a difference between the two compounds if applied to the living cell? Here again we encounter the fundamental importance of structure as mentioned above. Eserine is a tertiary amine and is, as a free base, lipid soluble. Prostigmine is a methylated quaternary ammonium salt and lipid insoluble. It appeared possible to assume that the inability of prostigmine to affect conduction in either nerve or muscle fiber, in contrast to eserine, may be due to the fact that the membranes surrounding the fibers are impervious to prostigmine. This assumption has been tested in the following way. Giant axons of Squid were exposed to high concentrations of prostigmine (10^{-2} M) for a certain period of time. After the exposure, the axoplasm was extruded and the presence of prostigmine was tested by adding the extruded axoplasm to cholinesterase. Whereas those anticholinesterases which affect the action potential are found in the axoplasm, prostigmine, in striking contrast, was completely absent (28).

This observation has obviously considerable significance. Acetylcholine and curare are, like prostigmine, methylated quaternary ammonium salts. The observation of Claude Bernard that curare acts exclusively on the nerve ending was for a century considered by many physiologists as evidence for a peculiar property of the nerve ending, suggesting that the mechanism of propagation of the nerve impulse differs fundamentally from that along nerve fibers. The hypothesis of neurohumoral or chemical transmission was based essentially on two facts. First, the excitability of synaptic junctions under certain conditions by acetylcholine, and, second, the appearance of acetylcholine in the perfusion fluid of these junctions under certain conditions, following nerve stimulation. The failure to affect conduction by acetylcholine, even in extremely high concentrations (20 gm per liter), was considered as evidence that the physiological function of acetylcholine, whatever it may be, is limited to the synaptic junctions (54). The failure of prostigmine, in contrast to all the other anticholinesterases to affect conduction and the limitation of its effect to the synaptic region, did obviously indicate that methylated quaternary ammonium salts can reach the active surface only at the synaptic region, especially the post-synaptic membrane. There the active membrane appears to be either less well or not at all protected by lipid. The limitation of the effect of acetylcholine applied externally to the synapse was therefore attributed to the difference in structure rather than to the underlying fundamental physico-chemical mechanism of propagation.

However, the massive doses of acetylcholine, applied without any effect, appeared so impressive that some doubts continued to persist whether the evidence based on the observation with prostigmine was sufficient. Our knowledge of the permeability of cell membranes is indeed limited. In view of the importance of this question, it appeared, therefore, desirable to offer direct evidence that the neuronal surface membranes are impervious to acetylcholine. The question has been tested in the following way: after exposure of nerves to acetylcholine labelled with N^{15} , the amount of N^{15} in the extruded axoplasm has been determined by means of a mass spectrometer and compared with the results obtained from a similar exposure to a tertiary amine labelled in the same way (55). The results are shown in Table I.

The tertiary amine penetrated easily and is nearly in equilibrium

after 60 min. The amount found inside after 25 min exposure to acetylcholine in high concentration (20 gm per liter) is negligible. 0.6 micromoles N (8.6 μ g) per gram axoplasm were found, which amounts to 0.67 per cent of the N outside. Even assuming that this would be the N of the choline, no physiological effect can possibly be expected if the penetration proceeds at such a low rate. One gram of these nerves can hydrolyze 2-4 mg of acetylcholine per hour, and therefore the infinitely small traces penetrating at any given moment would

TABLE I

Permeability of the membranes surrounding the stellar nerve of Squid to trimethylamine (TMA) and to acetylcholine (ACh) labelled with N¹⁵

The compounds contained 31 atom percent excess N¹⁵. After the exposure of the nerves to the compounds, the axoplasm was extruded and the N¹⁵ concentration determined.

COMPOUND	TIME OF EXPOSURE	N OUTSIDE	AXOPLASM = TOTAL N EXTRUDED		ATOM PERCENT EXCESS N ¹⁵	N DIFFUSED INTO AXOPLASM	PENETRATION
	min	μ moles/cc	mg	μ moles		μ moles	percent
TMA	15	20	134.3	69.2	0.333	5.5	30.5
	25	20	78.8	40.5	0.425	7.1	39.5
	60	20	149.4	77.1	0.916	15.3	84.5
ACh	25	100	137.0	70.7	0.038	0.6	0.67*

* Even this negligible amount of N found inside must be attributed to impurities present in the acetylcholine used. The 1430 μ g N per cc contained 55 μ g non-quaternary N. The N which penetrated (8.6 μ g per gram or 0.6 micromoles) is therefore equivalent to 18 per cent of the non-quaternary N outside. This is in the range to be expected.

be hydrolyzed before they could accumulate to a possibly effective concentration. Adding eserine would not change the situation because, in concentrations at which the cholinesterase would be inhibited to less than 80 per cent, the rate of hydrolysis would be still much too high compared with the rate of penetration. Still higher concentrations of eserine would abolish conduction even without additional acetylcholine being present.

However, even the negligible amounts found inside should not be considered as being quaternary ammonium salt. They must be attributed to the impurities of non-quaternary N present in the acetyl-

choline It was found that the alkali-distillable nitrogen in the acetylcholine bromide amounted to 0.6 per cent by weight (3.8 per cent of the total N). This is equivalent to 55 μg non-quaternary N in the 1430 μg N per cc of the solution used. The 8.6 μg N per gram of

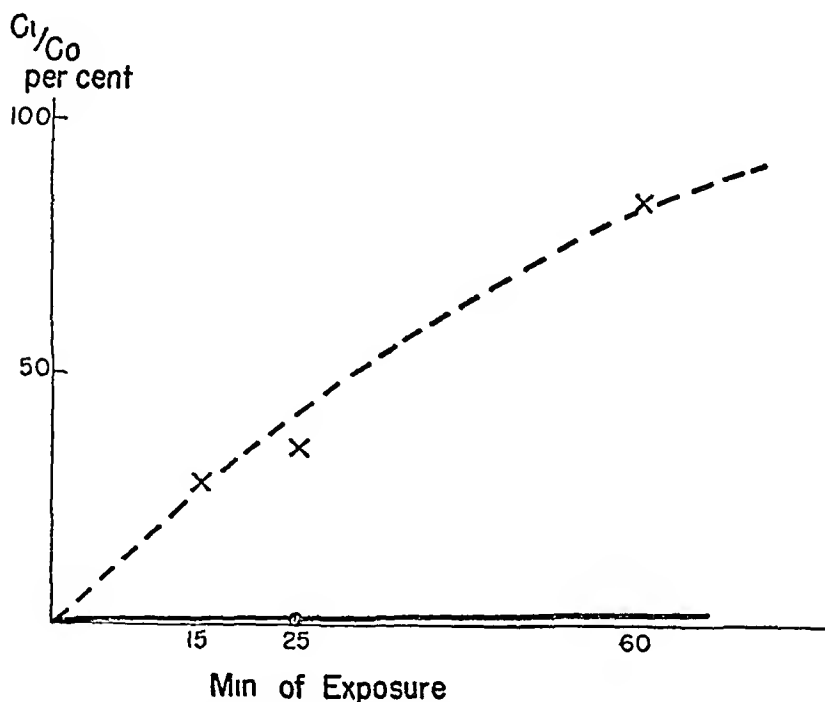


FIG. 6. RATE OF PENETRATION OF TRIMETHYLAMINE AND ACETYLCHOLINE LABELLED WITH N^{15} INTO THE INTERIOR OF THE GIANT AXON OF SQUID

The ratio of the concentration of the N of these compounds inside (C_i) to that outside (C_o) is plotted against the time of exposure in minutes. The dotted line indicates the rate of penetration of N (X) on exposure to trimethylamine (286 μg N per cc), the straight line, that of the N (O) found on exposure to acetylcholine (1430 μg N per cc of which 55 μg were non-quaternary N).

axoplasm (or 0.67 per cent of the outside concentration) must therefore be attributed to the non-quaternary impurities present in the acetylcholine used. The 8.6 μg N are equivalent to 18.0 per cent of the non-quaternary N outside. The rate of penetration of trimethylamine was, for the same length of exposure, 39.5 per cent. In view of the smaller gradient, the rate of penetration may have been actually

smaller. The agreement between the amount found, and that to be expected on the basis of the impurities present, is therefore entirely satisfactory. Fig 6 shows the difference of the rate of penetration of the 2 compounds.

The experiments show conclusively that the axonal surface membranes are impervious to acetylcholine, as was already suggested by

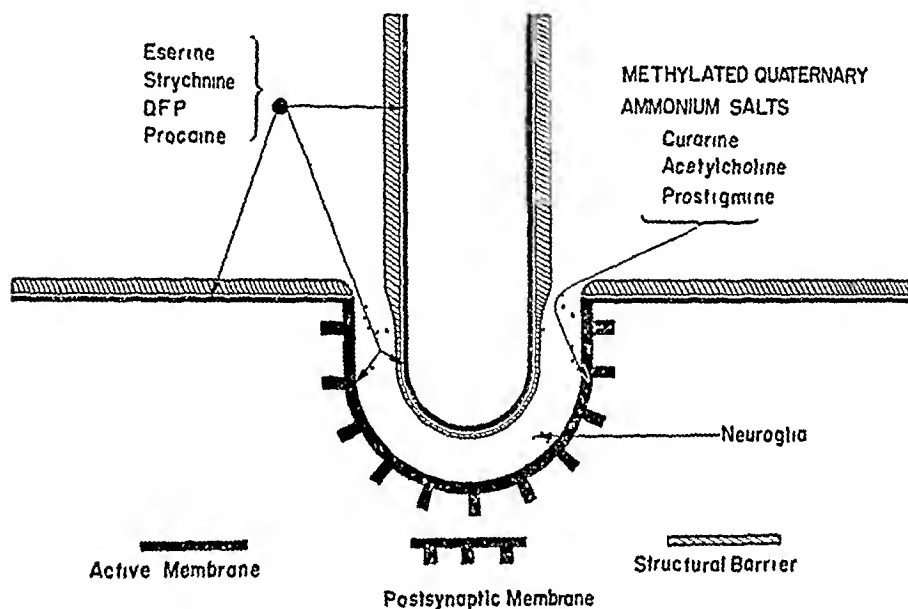


FIG 7 SCHEME OF THE NEUROMUSCULAR JUNCTION

A structural barrier protects nerve and muscle fiber against the action of methylated quaternary ammonium salts. These compounds act only on the post-synaptic membrane, which apparently is either less or not at all protected. Other compounds like eserine, DFP, strychnine, and procaine, being able to penetrate the structural barrier, act upon the active membrane of the nerve and muscle fiber, as well as upon synapse.

previous observations with prostigmine. They confirm the explanation given for the localized action of acetylcholine when applied externally to synaptic regions in contrast to its physiological rôle in conduction. More generally, the peculiar ability of the synapse to react to compounds which do not affect conduction is thus explained in terms of structure as illustrated in the scheme of Fig 7. It is the absence of the structural barrier at the post-synaptic membrane which

makes it possible to demonstrate the electrogenetic effect of acetylcholine in the electric organ

In view of these observations, the second fact on which the hypothesis of neurohumoral transmission was based, viz, the appearance of acetylcholine in the perfusion fluid of the synapse has also to be reconsidered. This appearance is obviously possible only there because of the absence of an insulating structural barrier, whereas the compound cannot leak out of stimulated nerve and muscle fibers. But even at the synaptic junctions, the ester does not appear under physiological conditions. It is an important fact that the ester appears only if the normal mechanism responsible for the rapid removal of the ester, viz, cholinesterase, is to a large degree inactivated by the presence of eserine. No trace of acetylcholine appears in absence of eserine. Even in presence of the drug, the amounts leaking out are infinitely small, one hundred-thousandth to one-millionth of that required to set up a stimulus, a discrepancy not easily explained in terms of chemical mediation.

The variations of structure may largely account for the great difficulties encountered and the many contradictions reported in applying the two criteria of chemical mediation to different types of synapses. The effects of compounds applied externally will vary in different types of tissue dependent upon the anatomical structure, the biochemical composition of the surrounding membranes and probably quite a few other accessory conditions. In view of the physicochemical properties of acetylcholine and similar methylated N-compounds, the difficulties will become nearly insurmountable in the study of brain and spinal cord which contain such large amounts of lipid and myelin. The painstaking efforts to demonstrate or to disprove the "cholinergic" nature of synapses in brain and spinal cord have led to a most unsatisfactory and confusing picture.

As pointed out so emphatically by von Muralt (56) in his book, the analysis of a cellular function, like the propagation of the nervous impulse, requires knowledge of structure, biochemistry, and biophysics. Each of these factors is of equal importance. Since the original approach to the elucidation of the rôle of acetylcholine in nerve activity has led to such an impasse, it should be expected that at least opponents of the idea of chemical mediation would try to

substitute these methods which proved to be inadequate. It is therefore surprising that Eccles, although accepting the necessity of a new approach, tries to disprove the rôle of acetylcholine in synaptic transmission by applying exactly the same type of methods that has been used for the support of the mediator theory. He exposed the frog's spinal cord to eserine in 10^{-4} M concentration and to prostigmine in $3-6 \times 10^{-5}$ M concentration. No effect on synaptic transmission was obtained (57, 58). Eccles assumes that the cholinesterase in the spinal cord under his experimental conditions, was completely inactivated by the eserine and excludes, therefore, any rôle of acetylcholine in the synapses of the spinal cord. The assumption that cholinesterase activity was completely abolished was not tested experimentally but was based on the figure reported in the literature to be necessary for inhibiting cholinesterase in solution or suspension. But in Eccles' type of experiments, it is necessary to take into account structural barriers, penetration rates, the kinetics of the enzyme inhibition, the excess of the enzyme, and a number of other factors upon which the effect of drugs applied to cells and organs may depend. In order to inactivate cholinesterase to a sufficiently high degree for obtaining interference with conduction in a single fiber preparation, the giant axon of Squid, a concentration of 10^{-3} M eserine, must be used. This preparation has a lipid layer only a few micra thick. The eserine is applied at a pH of 8.2 to 8.4, the pH of sea water. At this pH, a greater fraction of eserine is undissociated and therefore penetrates more readily as free base than at a pH of 7.2 used in Eccles experiments at which the compound is nearly completely dissociated. The reason for the failure to obtain an effect in the spinal cord, especially with the low concentrations used, is then obvious.

In contrast to the conflicting results obtained when the "cholinergic" nature of nerves is tested by the criteria mentioned, the approach by the study of the enzymes connected with acetylcholine metabolism and their correlation with function did not encounter comparable difficulties. All facts support the assumption of the generality of the rôle of acetylcholine in all nerve tissue, including that of brain and spinal cord. The presence of large amounts of cholinesterase in brain and spinal cord studied extensively during the years 1937 to 1939 (59-62) suggested that the ester may have the same rôle there as in

the periphery The time when the high concentration of the enzyme appears in the brain during embryonic development coincides with the beginning of function (61, 62) The most striking evidence for this coincidence was obtained in studies on brain and spinal cord of sheep embryos suggested by Sir Joseph Barcroft (63) The discovery of choline acetylase made possible the demonstration of the high rate of acetylcholine formation in brain as well as in all other nerve tissues Finally, the observations reported with DFP have shown that cholinesterase activity in brain is indispensable for life

As pointed out before, the two fundamental facts on which the theory of neurohumoral transmission was based originally appear today in a new light The ability of the synapse to react to acetylcholine applied externally has been explained on the basis of structural difference between synapse and axon No good evidence exists that the appearance of the ester in the perfusion fluid of synaptic region is a physiological event Thus it appears imperative to reconsider the theory of neurohumoral transmission and to discuss whether or not the original interpretation of the observations of Loewi and Dale has to be changed

At the symposium on the synapse in 1939, Erlanger emphasized that many properties considered to be peculiar to the synapse can be obtained in the axon, like spatial summation, one-way transmission, latency, and transmission of the action potential across the non-conducting gap (3) It appears from these studies that the electrical signs of activity do not justify the assumption that the mechanism of transmission across synapses differs fundamentally from that along axons During the last ten years, extensive investigations have been made on the electrical characteristics of transmission across artificial ephapses and natural synapses by a great number of investigators, like Arvanitaki, Bullock, Eccles, Granit and Skoglund, and many others From the various investigations, considerable evidence has accumulated that the propagating agent across the synapse is the flow of current According to Eccles (64), impulses in a pre-synaptic nerve fiber generate a current which gives a diphasic effect on the synaptic region of the post-synaptic cell This current produces initially an anodal focus with cathodal surround, followed by a more intense cathodal focus with anodal surround The cathodal focus sets up a

local response from which a catelectrotonus spreads over the post-synaptic cell membrane. This catelectrotonus, the end-plate potential, sets up a propagated impulse in the post-synaptic cell as soon as a certain threshold is reached. The sequence of events is similar to that observed on artificial synapses (ephapses) and on a single unit preparation of the synapse, the Squid stellate ganglion (Bullock (65)).

In view of all this evidence accumulated, it appears more likely to assume that the rôle of acetylcholine at the synapse is the same as in the axon. Release and removal of acetylcholine must be essential events in the changes of the presynaptic membrane during the flow of current across the synaptic region and in the post-synaptic membrane generating the end-plate potential. It would be difficult to picture these currents as being different in nature from those in the axon.

There is experimental support for the assumption of a high rate of acetylcholine metabolism in the post-synaptic membrane. Observations, on the cholinesterase concentration at the motor end-plate following the section of the motor nerve, have revealed that the high concentration at the motor end-plate decreases by less than a third within 3 to 4 weeks (66-67). It then remains stable for many months. Most of the enzyme present in high concentration at the motor end-plate is apparently localized in the post-synaptic membrane, the site of the end-plate potential, an exclusively muscular element. Less than one-third may be localized in the pre-synaptic membrane. Another indication of the rôle of acetylcholine in the end-plate potential is the direct proportionality between voltage and cholinesterase in the electric tissue, since the discharge there may be considered as homologous to the end-plate potential.

In view of the high rate of metabolism in the post-synaptic membrane during the development of the end-plate potential, it may be expected that in presence of eserine not all the acetylcholine released is hydrolyzed with the usual speed. A fraction of the ester may therefore leak and be found in the perfusion fluid. Much more convincing evidence than that available today would be necessary for the acceptance of the idea that acetylcholine assumes at the synapse a function entirely different from that in the axon, i.e., is released from the nerve ending, penetrates the neuroglia and acts on the post-synaptic membrane, thus substituting the flow of current as chemical mediator.

All indications favor the assumption that the mechanism of the transmission of the impulse across synapses is fundamentally identical with that along axons, the transmitting agent being the flow of current in both cases. This interpretation does not minimize the importance of the observations of Loewi and Dale, but harmonizes them with the progress of our knowledge concerning the structure, the biochemical data, and the electrical signs of nerve activity¹

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¹ After this lecture had been prepared, Eccles' review appeared in the *Ann Rev. of Physiol*, 1948. Eccles rejects resolutely the possibility of any rôle of acetylcholine in conduction. His opposition is based on the same objections which were presented at the symposium in 1946. Since all these objections have been extensively discussed, no additional comment appears necessary.

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DISCUSSION

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Much study in recent years has fortified the so-called chemical theory of the activity of nerves. However, many facts must be harmonized with the view and many problems appear if the view is accepted. A few of the problems are indicated in the following discussion in the hope that apparently discordant facts may be integrated with the concept and that some lines of further inquiry may be indicated.

The review just presented has highlighted the studies which support the hypothesis that acetylcholine (ACh) action is a primary event in impulse conduction in nerve. It has been claimed also that ACh plays a similar part in other excitable tissue, such as muscle, thus broadening the hypothesis to state that ACh is the universal exciting agent. The

wide distribution of a hydrolytic enzyme (or enzymes) of marked activity toward choline esters, the occurrence of ACh in tissues, and an enzyme for its synthesis lend support to the hypothesis. The concentration of the enzyme cholinesterase (ChE) in those portions of organs considered important for excitation further fortifies the hypothesis.

At the outset, it may be useful to recall that throughout the review emphasis is placed upon the ChE mechanism and that reduction of this enzyme to a certain level of concentration brings about failure of conduction. It should be emphasized that thereby attention has been centered on the problem of removal of ACh after it has performed its function. Hence, it may be said that, strictly speaking, ChE is not essential for conduction but rather for repolarizing the nerve fiber following the action of free ACh, thus resetting the "trigger" for the next impulse. This consideration follows if we are to explain the blocking effect of anticholinesterase compounds by the accumulation of ACh, such an accumulation would allow passage of current leading to unrelieved depolarization of the membrane. In this connection, since ChE inhibitors such as di-isofluorophosphate (DFP) appear to combine with the chemical grouping in ChE responsible for splitting ACh, it is possible that the "receptor" on the effector is of like chemical configuration. Hence, the receptor might likewise react with DFP, though undoubtedly in a much lower order of reactivity, thus far, such a reaction has not been observed. The experiments of Toman do not support the supposition that anticholinesterase agents block conduction by permitting ACh to accumulate to levels which result in sustained depolarization. This investigator found no correlation between changes in conduction and in the demarcation potential during the course of development of nerve block by DFP. It is important that Toman's findings be confirmed and extended. Recently, utilizing an organ not requiring hydrolysis of ACh for sustained activity, the submaxillary gland of the cat, Riker (personal communication) has observed that spontaneous salivary activity appears when the ChE of the gland is reduced to 10% and that between this value and zero, the rate of salivary flow is proportional to the reduction in ChE. In frogs' muscle we (1) have found that of certain compounds structurally related to DFP only those inactivating ChE rendered the muscle inexcitable. The correlations between nerve action potential and ChE

activity which have been reported are therefore valid only as reflections of the capacity of nerve to revert to polarization after activity. It is relevant to indicate here that such correlations are best studied in single fiber preparations in which all-or-none response to a stimulus is clearly seen.

This leads us to inquire about the release of ACh, in an active form, from excitable tissue receiving a stimulus. Such "release" must be an extraordinarily rapid process, probably much more so than the removal of ACh, in itself an event of great rapidity. If ACh is the agent responsible for the propagated disturbance, its "release" therefore appears to be the essential event in excitation and in the propagation of the impulse, ChE is in this light essential for restoration of the membrane in order that repeated and sustained activity of nerve be possible.

Concerning the part which ACh may play in central synaptic transmission, we were unable to duplicate Bremer's experiments in which small amounts of ACh by intracarotid injection produced increased cortical potentials. We have observed, however, that atropine prevents or annuls the convulsant effects of DFP as seen by means of potential patterns from the brain in experimental animals (2). This is accomplished with quantities of atropine having little effect upon normal potential patterns. The effect appears specific because even far larger quantities of atropine have no effect upon the potential patterns induced by such powerful CNS stimulants as pentamethylene tetrazol, commonly called metrazol. It follows that if ACh is the common denominator in axonal conduction and in certain portions of synaptic transmission, then atropine might be expected to prevent or interrupt with equal facility the perpetuation of the effects of metrazol along a chain of neurones regardless of the manner in which metrazol initiates its effects. Further study is obviously in order here to harmonize the discrepancy. One might speculate that atropine prevents ACh action on an area on a postsynaptic neurone, such as on the muscle end-plate, which is normally excited by flow of current generated at the presynaptic site by ACh.

According to the ACh-ChE hypothesis, adenosine triphosphatase constitutes one of the links in the chain of the energy feeder system for maintaining a supply of ACh via choline acetylase. The large dif-

ference between the turnover members of ChE and adenosine triphosphatase is cited in support of the primacy of ACh over adenosine triphosphatase. It should be borne in mind that despite this difference, adenosine triphosphatase activity must keep pace with release and hydrolysis of ACh during periods of sustained nerve activity.

It is interesting to consider some fundamental properties of nerve as well as synapses in the light of the ACh mechanism. Latency may be considered the time necessary for release, among other events, of sufficient ACh for depolarization and setting up a propagated response. Incidentally, ACh release must take place prior to and possibly during the rise of the spike of the action potential, 0.1 to 0.3 millisecond in some cases, or even more quickly. Facilitation, temporal summation, and recruitment may be akin to the local changes prior to excitation as described by Bronk and co-workers, these changes may be regarded as the effects of sub-threshold concentrations of ACh, finally resulting in the initiation of propagated disturbances as the concentration of ACh rises. Adaptation presents a more difficult problem, for it is unknown how it is related to ChE content, ACh store, and ACh restitution from the hydrolysis products.

It would be useful to obtain a formulation of the temporal and quantitative relation of ACh release, removal, and restitution to the several phases of the nerve action potential as it may be visualized at present: latent period, spike potential, and threshold changes (supernormal period, refractory period).

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II QUATERNARY AMMONIUM IONS AND SODIUM IONS IN NERVE PHYSIOLOGY¹

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The research presented in this report was begun with the purpose of ascertaining why tetraethyl-ammonium can substitute for sodium in certain important aspects of nerve function while neither tetramethyl-ammonium nor choline is able to substitute for sodium.

As was originally demonstrated by Overton ('02), and was repeatedly confirmed by the present writer (Lorente de Nó, '44, '47), sodium ions play an essential rôle in nerve physiology. A frog nerve deprived of sodium, i.e., a frog nerve that is being kept in a sodium-free medium, ultimately becomes inexcitable. The inexcitability is reversible, since the nerve fibers rapidly regain the ability to conduct impulses after sodium ions are made available to them.

Overton used sugar solutions as sodium-free media, for a number of reasons, however, it is more convenient to use solutions of certain quaternary ammonium ions. The chlorides of several quaternary ammonium ions can be used at the 0.11 M concentration to prepare sodium-free media that are "inert," i.e., that do not cause a depolarization of the nerve fibers. In the research presented here use has been made of the chlorides of tetramethyl-ammonium (fig. 1, I), ethyltrimethyl-ammonium (fig. 1, II), choline (fig. 1, VI) and in particular of diethanol-dimethyl-ammonium (fig. 1, X).

In sugar solutions, or in solutions of the inert quaternary ammonium ions, the fibers of fast conduction begin to become inexcitable after 8–10 hours, and usually after 14–16 hours the fibers of slow conduction also are unable to conduct impulses. Complete restoration by transfer of the nerve to Ringer's solution can be obtained even after the nerve has been kept in a sodium-free solution of an inert quaternary ammonium ion for as long as 24–36 hours.

¹ This report is a summary of an extensive paper "On the effect of certain quaternary ammonium ions upon frog nerve" that is in the course of publication in the *Journal of Cellular and Comparative Physiology*. The figures included in this report have been reproduced by permission of the editors of the *Journal of Cellular and Comparative Physiology*.

To make sodium ions available to the nerve, either 0.11 M sodium chloride or Ringer's solution can be used since potassium and calcium ions, neither at the concentrations at which they are present in Ringer's solution nor at any other concentration, are able to substitute for sodium. If the nerve has been kept in a sodium-free medium for a long

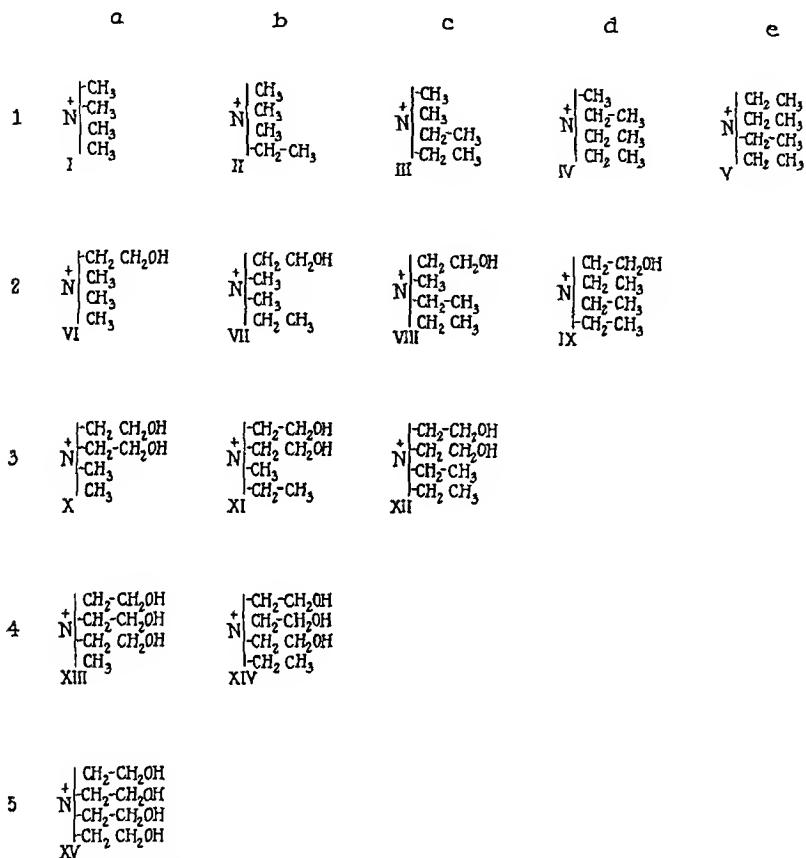


FIG. 1. THE 15 POSSIBLE COMBINATIONS OF METHYL ($-\text{CH}_3$), ETHYL ($-\text{CH}_2-\text{CH}_3$) AND ETHANOL ($-\text{CH}_2-\text{CH}_2\text{OH}$) GROUPS IN QUATERNARY AMMONIUM IONS

The Roman numerals I to XV are used in the text to identify the individual ions

period of time, complete restoration by sodium is not obtained unless the concentration of sodium in the external medium of the nerve fibers is approximately 0.1 M. Thus, since the problem under investigation has been the ability of quaternary ammonium ions to substitute for sodium, the concentration of the solutions of "restoring" quaternary ammonium ions always has been 0.11 M.

In sharp contrast with the behavior of nerves kept in a 0.11 M solution of the chloride of one of the inert quaternary ammonium ions, nerves kept in a 0.11 M solution of tetraethyl-ammonium chloride become only partially inexcitable. The fibers of fast conduction (A fibers in Erlanger and Gasser's classification) lose their ability to conduct impulses approximately in the same manner as in other sodium-free media, the fibers of slow conduction, however, remain able to conduct impulses practically as long as they would in Ringer's solution. It is convenient to call Et fibers those fibers which remain excitable in 0.11 M tetraethyl-ammonium chloride. The Et class includes the majority, if not all the fibers of the B and C groups of Erlanger and Gasser's classification. The Et class, therefore, includes both myelinated and unmyelinated fibers and is numerically the more important class of fibers.

On the other hand, if after a nerve has become inexcitable in a 0.11 M solution of one of the inert quaternary ammonium ions, or, more generally stated, in a sodium-free medium that does not cause a depolarization of the nerve fibers, the nerve is transferred to a 0.11 M solution of tetraethyl-ammonium chloride the Et fibers regain their ability to conduct impulses practically as rapidly as they would in 0.11 M sodium chloride. Tetraethyl-ammonium ions, therefore, are able to substitute for sodium in an important aspect of nerve function.

In view of this situation, the first assumption that comes to one's mind is that the ethyl group, when it is attached to tetravalent nitrogen, plays a specific rôle in nerve physiology. The assumption has proven to be incorrect, nevertheless, it played the rôle of a useful working hypothesis.

In order to test the validity of the hypothesis, experiments were conducted with the use of the quaternary ammonium ions listed in figures 1 and 6. Let us consider first the ions listed in figure 1. As can readily be noted those 15 ions are the 15 possible combinations of methyl ($-\text{CH}_3$), ethyl ($-\text{CH}_2-\text{CH}_3$) and ethanol ($-\text{CH}_2-\text{CH}_2\text{OH}$) groups in quaternary ammonium ions. Three of the ions (I, V and VI) are commercially available (Eastman Chemicals), the other 12 were synthesized in the laboratory by well-known methods.

The description of the experiment illustrated by figure 2 will also serve to outline the technique used in the majority of the experiments to be mentioned in this report. The nerve was kept in a large volume

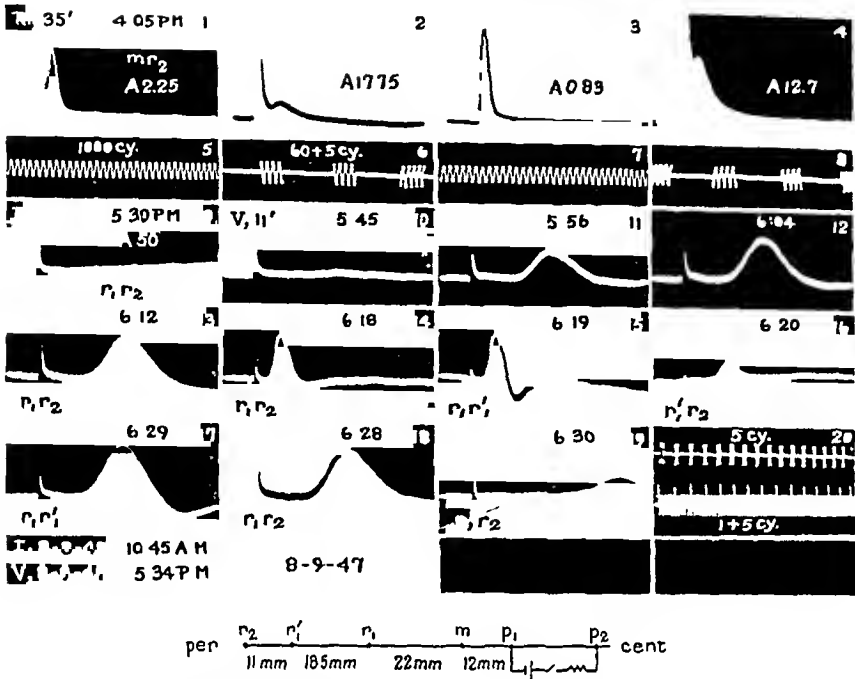


FIG 2 RESTORATION OF THE EXCITABILITY OF NERVE FIBERS DEPRIVED OF SODIUM IN A 0.11 M SOLUTION OF TETRAMETHYL-AMMONIUM CHLORIDE (FIG 1, I)

The diagram at the bottom indicates the interelectrode distances used. The central segment (mp_2) was treated with Ringer's solution (R), the peripheral segment (mr_1') with a 0.11 M solution of tetraethyl-ammonium chloride (V), no restoring solution was applied to the $r_1'r_2$ segment.

1 to 4, restoration of the central segment. 1 and 3 spikes of fibers of fast conduction recorded at point m, 2 and 4 spikes of fibers of slow conduction recorded at point m. Record 3 was obtained at 5.26 p.m. The time lines 5 to 8 apply to the corresponding records.

9 to 19, restoration of the peripheral segment. The lower time line in record 20 applies to records 14 to 16, the upper line to the other records of the series 9 to 19. Lack of conducted response before the application of the restoring solution. Note the upward drift of the base line, similar drifts appear in other records (13, 15, 16) as well as in records of other figures. The drifts were referable to slow variations in the potential of the recording, silver-silver chloride electrodes, for this reason they were greatest immediately after each change in the solution in contact with the nerve, even when they were great they did not interfere with the observation of conducted responses.

In this and similar figures the composition of the solution in contact with the nerve is given on the upper left corner of the 1st record of each series (cf records

of a sodium-free medium (0.11 M tetramethyl-ammonium chloride) for slightly over 29 hours, at the end of this time the nerve was mounted in a moist chamber resting upon the electrodes of the stimulating and recording circuits. The arrangement of the electrodes is indicated in the diagram in figure 2. The central segment of the nerve (fig. 2, below, mp₂) was placed in contact with Ringer's solution and the recovery of this segment was followed by recording the action potential of impulses initiated at electrode p₁ with the oscillograph connected to electrodes m and r₂. Rectangular pulses of current were used to initiate the impulses. Records 1 to 4 of figure 2 illustrate two stages of the recovery of the fibers of fast conduction (records 1 and 3) and of those of slow conduction (records 2 and 4). One hour was allowed for the recovery of the central segment. In experiments in which the nerves have been deprived of sodium for long periods of time the fibers of slow conduction begin to recover their excitability in the presence of sodium earlier than the fibers of fast conduction. As a rule, nerve fibers begin to be able to conduct impulses within 6-8 minutes and the recovery becomes practically complete in about 1 hour.

Tests were then made, with the oscillograph connected to electrodes r₁ and r₂, of the ability of the impulses initiated at electrode p₁ to propagate themselves into the peripheral segment. Record 9 (fig. 2) displays only the shock deflection, indicating that the segment of the nerve fibers that was still deprived of sodium was unable to conduct impulses. The peripheral segment of the nerve (fig. 2, below, mr₂) was

1, 9, 10), the Roman numerals identify the ions listed in figures 1 and 6, R indicates Ringer's solution. Usually, the interval of time elapsed since a new test solution was applied is also given, for example V, 11' in record 10 indicates that the 0.11 M solution of tetraethyl-ammonium chloride had been placed in contact with the nerve 11 minutes before record 10 was obtained. The time at which the records were obtained is given with the records, when no time is given the record was obtained shortly after the preceding one. When the interval of time elapsed between successive records is significant it is given in seconds on the upper left corner of the records. For example, record 9 of figure 4 was obtained at 11.32 p.m. and record 10, 10 seconds later. The amplification is given with the 1st record obtained at a new amplification, in mm deflection per mv input. Thus, A 2.25 indicates that 1 mv corresponds to a deflection of 2.25 mm, when the records are reproduced so that the width of the records measures 32 mm.

then placed in contact with a sodium-free "restoring" solution, that, in the experiment which is now under consideration, was a 0.11 M solution of tetraethyl-ammonium chloride. A few Et fibers became able to conduct impulses after the tetraethyl-ammonium ions had acted upon the nerve for 11 minutes (record 10), i.e., in approximately the same interval of time that would have been necessary for the recovery to begin in the presence of sodium. The number of responding fibers increased with advancing time (records 11, 12, 13), and after one hour the recovery became practically complete (records 17, 18).

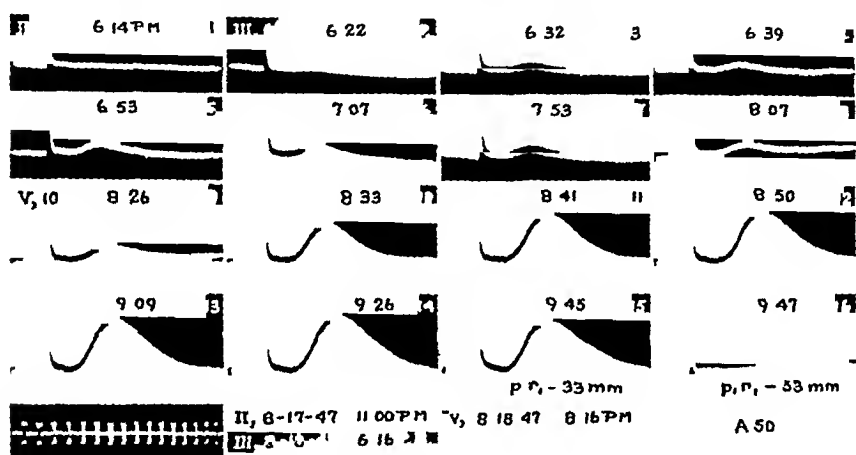


FIG 3 Restoration of the ability to conduct impulses to fibers of class Et, rendered inexcitable in a solution of ethyl-trimethyl-ammonium ions (record I) by dimethyl-diethyl-ammonium ions (records 2 to 8) and by tetraethyl-ammonium ions (records 9 to 16). The conduction distance for records 1 to 15 was 33 mm, that for record 16, 53 mm. The amplification was constant (A 50).

The experiments of the series done with the use of the ions listed in figure 1 have shown (1) that those ions which have no ethyl group or only 1 ethyl group cannot substitute for sodium, while (2) those ions which have 2 or more ethyl groups substitute for sodium and restore the excitability of Et fibers. The ability to substitute for sodium increases with the number of ethyl groups attached to nitrogen. Figures 3 to 5 place this important fact in evidence.

In the experiment illustrated by figure 3 the nerve was rendered inexcitable in 0.11 M ethyl-trimethyl-ammonium chloride (fig 1, II). Restoration was effected with a 0.11 M solution of dimethyl-diethyl-

ammonium chloride (fig 1, III) The recovery began very promptly, since as is shown by record 2 a certain number of fibers became able to conduct impulses after the dimethyl-diethyl-ammonium ions had acted

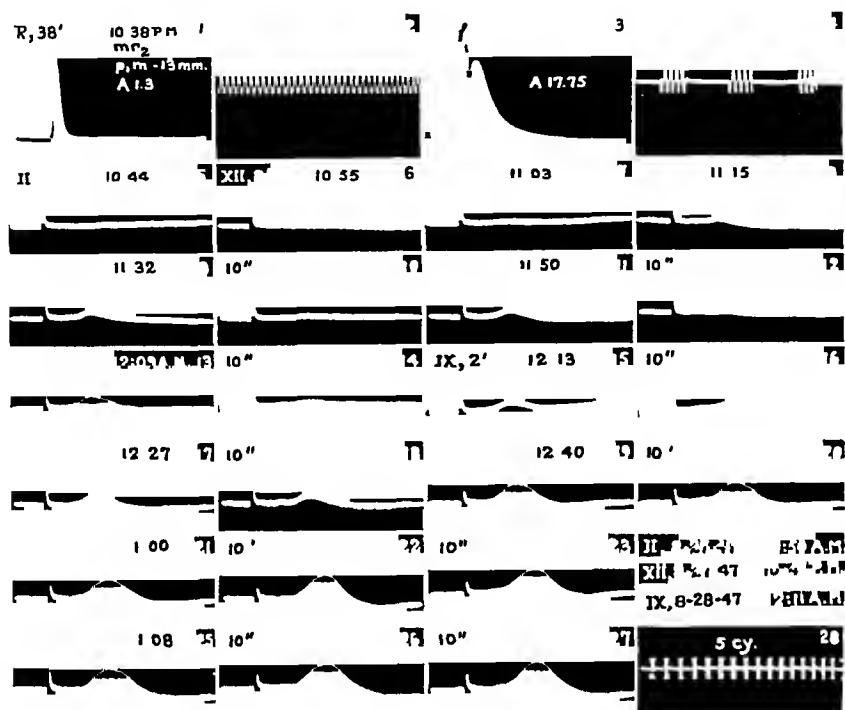


FIG 4 RESTORATION OF THE EXCITABILITY OF A NERVE RENDERED INEXCITABLE IN 0.11 M ETHYL-TRIMETHYL-AMMONIUM CHLORIDE

1 and 3, responses recorded at point m, 38 minutes after the central segment had been placed in contact with Ringer's solution, time line 2 (1000 cv) applies to record 1, time line 4 (60 + 5 cy) to record 3

5, absence of conducted response in the peripheral segment of the nerve, 6 to 14, restoration by diethanol-diethyl-ammonium, 15 to 27, enhancement of the restoration by ethanol-triethyl ammonium The amplification (A 50) and the sweep speed (record 28) were constant for records 5 to 27

upon the nerve for 6 minutes The number of conducting fibers increased progressively with advancing time (records 3 to 6), nevertheless, after the dimethyl-diethyl-ammonium ions had acted upon the nerve for 50 minutes, the response ceased to grow (records 7, 8), thus indicating that those ions were able to restore the excitability of only

a relatively small number of Et fibers. Tetraethyl-ammonium ions proved to be able rapidly to increase the number of conducting fibers (records 9 to 16)

In the experiment illustrated by figure 4 the nerve was rendered inexcitable also in a 0.11 M solution of ethyl-trimethyl-ammonium ions. Records 1 and 3 present spikes recorded at point m (cf fig 2, below) during the restoration of the central segment by Ringer's solution. Record 5 places the fact in evidence that no nerve impulse was able to propagate itself beyond point m into the peripheral segment. The restoration of this segment was begun with 0.11 M diethanol-diethyl-ammonium chloride (fig 1, XII). The diethanol-diethyl-ammonium ions were able to effect only a partial recovery, since only a discrete number of Et fibers became able to conduct impulses (records 6, 7, 8, 9, 11, 13), in addition, the records of the pairs 9, 10, 11, 12 and 13, 14, that were obtained at 10-second intervals, show that the restored fibers were exceedingly susceptible to fatigue. A marked improvement of the recovery was effected by means of the ethyl homologue of choline. This ion (fig 1, IX), that contains 3 ethyl groups, rapidly increased the number of conducting fibers, in addition, it enabled the restored fibers to conduct impulses at 10-second intervals with but little fatigue (records 15 to 27).

Figure 5 illustrates in a more direct manner the effect of replacing the methyl groups of choline by ethyl groups. The experiment was done with the 2 sciatic nerves of a bullfrog. The nerves were allowed to become inexcitable in a 0.11 M solution of choline chloride. Records 1 to 15 and 25, 26 illustrate the final stages of the development of inexcitability. At the time when records 1 and 2 were obtained, nerve I had been in the choline chloride solution for approximately 14 hours. No fiber of fast conduction was able to conduct impulses, but a significant number of fibers of slow conduction still was excitable. Records 3 to 15 show that the number of conducting fibers decreased with advancing time, until at the time when records 13 to 15 were obtained only a few fibers were able to conduct impulses. The peripheral segment of the nerve was then placed in contact with a 0.11 solution of ethanol-triethyl-ammonium ions (fig 1, IX) with the result that the Et fibers rapidly regained their excitability (fig 5, 13 to 24). In the case of nerve II the restoration (fig 5, 27 to 36) was effected by means of n-propyl-triethyl-ammonium ions (fig 6, XVII).

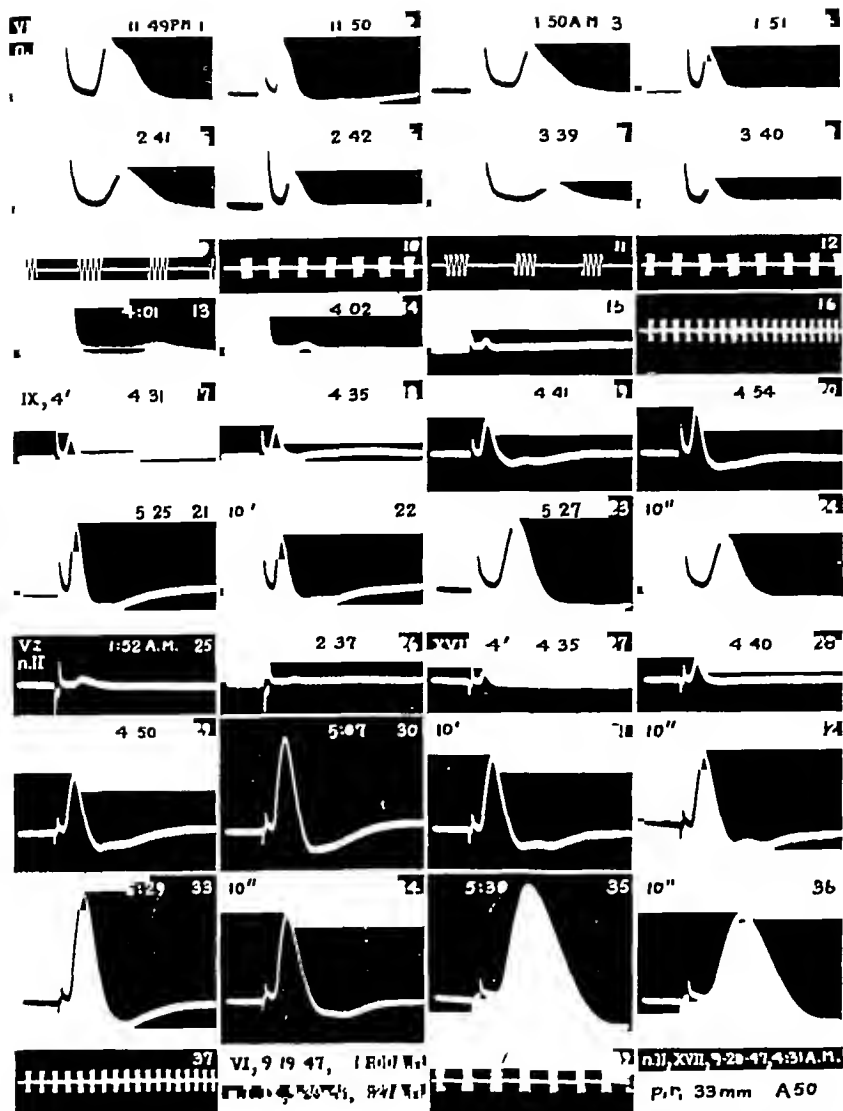


FIG 5 RESTORATION OF THE EXCITABILITY OF PAIRED NERVES RENDERED INEXCITABLE IN 0.11 M CHOLINE CHLORIDE

The observations were begun after the central segments had performed complete recoveries in Ringer's solution

1 to 15, terminal phases of the development of inexcitability of fibers of slow conduction in nerve I (n I), time lines 9 and 11 correspond to records 1, 3, 5, 7, and 13, times lines 10, 12 to records 2, 4, 6, 8 and 14, time line 16 to record 15 17 to 24, restoration of nerve I by ethanol-triethyl-ammonium ions Records 17 to 22 were obtained with the sweep speed of record 16, and records 23, 24 with the speed of record 12

25, 26, terminal phases of the development of inexcitability of the fibers of slow conduction of nerve II (n II), 27 to 36, restoration of nerve II by n-propyl-triethyl-ammonium ions Records 25 to 34 were obtained with the sweep speed of record 37, records 35, 36, with that of record 39 The conduction distance (33 mm) and the amplification (A 50) were constant

Additional information was obtained in experiments done with the use of the ions listed in figure 6. Four of those ions are commercially available (V, XXII and XXIII, Eastman Chemicals, XXV, Merck), the others were synthesized in the laboratory by well-known methods.

It will be noted that all the ions listed in columns a, b and d of figure 6 have 3 ethyl groups, all those ions are able to substitute for sodium, in so far as they restore the excitability of Et fibers that have become inexcitable in a sodium-free medium. In view of this fact it may be assumed, with some assurance, that in general quaternary ammonium ions having 3 ethyl groups will be able to substitute for sodium. The assumption, however, must be submitted to experimental test in each individual case, since the nature of the 4th group attached to nitrogen contributes to determining the properties of the ion, indeed, the 4th group may play a very important rôle.

For instance, in the series of ions listed in column a of figure 6 important changes in properties result from lengthening the carbon chain of the 4th group from 1 to 6 atoms.

(a) The ability of ion V to restore the excitability of Et fibers deprived of sodium is considerably greater than that of ion IV. The restoring abilities of ions V, XVI and XVII are approximately equal, and that of ion XVIII is only slightly weaker than that of ion V, but the properties of ion XIX are quite different from those of ion V. It is true that ion XIX is able to restore the excitability of a number of Et fibers, but if it is allowed to act upon the nerve for longer than 60-90 minutes the Et fibers again become inexcitable, with the noteworthy peculiarity that the change produced by ion XIX in the Et fibers is not reversible by sodium, even though sodium is able to restore the excitability of the fibers of fast conduction.

(b) In a frog nerve kept in a 0.11 M solution of tetraethyl-ammonium chloride the A fibers maintain their membrane potential at the normal level, while nerves kept in 0.11 M solutions of the other ions listed in column a undergo a progressive depolarization. The depolarizing action of ion XVI is weak, while that of ion IV is quite strong, and in the series of ions XVI to XIX the depolarizing action increases with increasing length of the carbon chain of the 4th group.

In reference to ions XX and XXI it will be sufficient to mention that ion XX is a very effective, restoring ion, but the restoring ability of ion XXI is quite limited.

A similar difference exists between the properties of ions IX, the ethyl homologue of choline and XXIV, the ethyl homologue of acetyl-

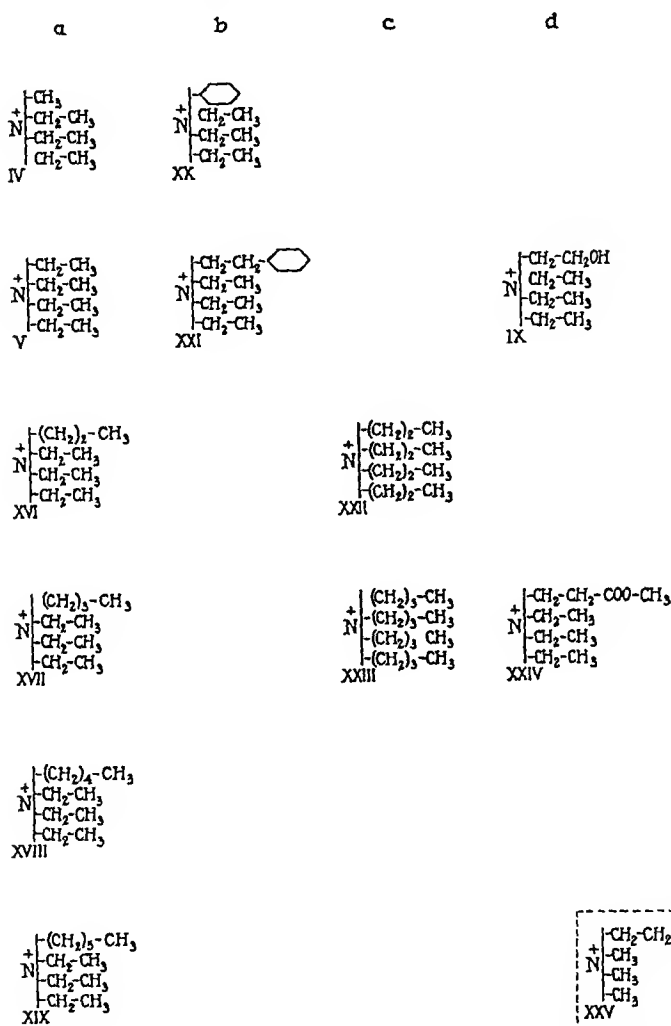


FIG 6 QUATERNARY AMMONIUM IONS OF THE RESTORING TYPE

The ions in columns a, b and d have 3 ethyl groups, those in column c have no ethyl groups. The formula of the acetylcholine ion (XXV) is included solely for the purpose of comparison, since acetylcholine belongs to the inert group

choline (XXV). Ion IX is a powerful restoring agent (fig 5, 17 to 24), but the restoring ability of ion XXIV is rather small.

In the experiment illustrated by figure 7 the nerve was rendered inexcitable in a 0.11 M solution of diethanol-dimethyl-ammonium

rapidly increased the number of responding fibers (records 13 to 16), later, the response decreased progressively, an effect that was referable to certain peculiarities of the action upon nerve of phenyl-triethyl-ammonium, which cannot be discussed in this brief report

An important fact is that restoration of excitability of Et fibers deprived of sodium can be effected by means of ions XXII (tetrapropyl-ammonium) and XXIII (tetrabutyl-ammonium), i.e., by means of quaternary ammonium ions that do not contain ethyl groups. Also important is, probably, the fact that the restoring ability of ion XXIII is greater than that of ion XXII.

The experiment illustrated by figure 8 was done with the 2 sciatic nerves of a bullfrog. The nerves were rendered inexcitable in 0.11 M diethanol-dimethyl-ammonium chloride. The restoration by tetra-n-butyl-ammonium ions began approximately as fast as if tetraethyl-ammonium ions had been used. It is true that no nerve fiber had regained its ability to conduct after 7 minutes (record 3), but a significant number of fibers regained their ability to conduct in 14 minutes (record 4). The restored response rapidly increased in size to become maximal in 19 minutes (record 9). As is shown by records 5 to 8, the restored fibers were able to conduct impulses at 10-second intervals with but little fatigue. Soon thereafter, the restored response began to decrease (records 9 to 12, 13 to 15), and 55 minutes after the restoring solution had come in contact with the nerve the Et fibers were again inexcitable. The explanation of this fact is that ultimately tetra-n-butyl-ammonium ions cause a depolarization of the nerve fibers.

The restoring action of tetra-n-propyl-ammonium ions began at a very low rate (fig. 8, 19 to 24), after 1 hour the response still was quite small and the restored fibers were very susceptible to fatigue (records 25 to 28). The nerve was then placed in contact with the 0.11 M solution of tetra-n-butyl-ammonium chloride, whereby a rapid enhancement of the recovery was produced. In 7 minutes the tetra-n-butyl-ammonium ions were able to increase the number of conducting fibers (cf. records 25 and 29) and markedly to diminish their fatigability (records 29 to 32). The restored response still increased in size during the following 10 minutes (records 33 to 36), thereafter the response decreased (records 37 to 39) and soon all the nerve fibers proved to be inexcitable (record 40).

Since ions XXII and XXIII are able to restore the excitability of Et fibers deprived of sodium, it is clear that the restoring ability of ions that contain 2 or more ethyl groups cannot be explained in terms

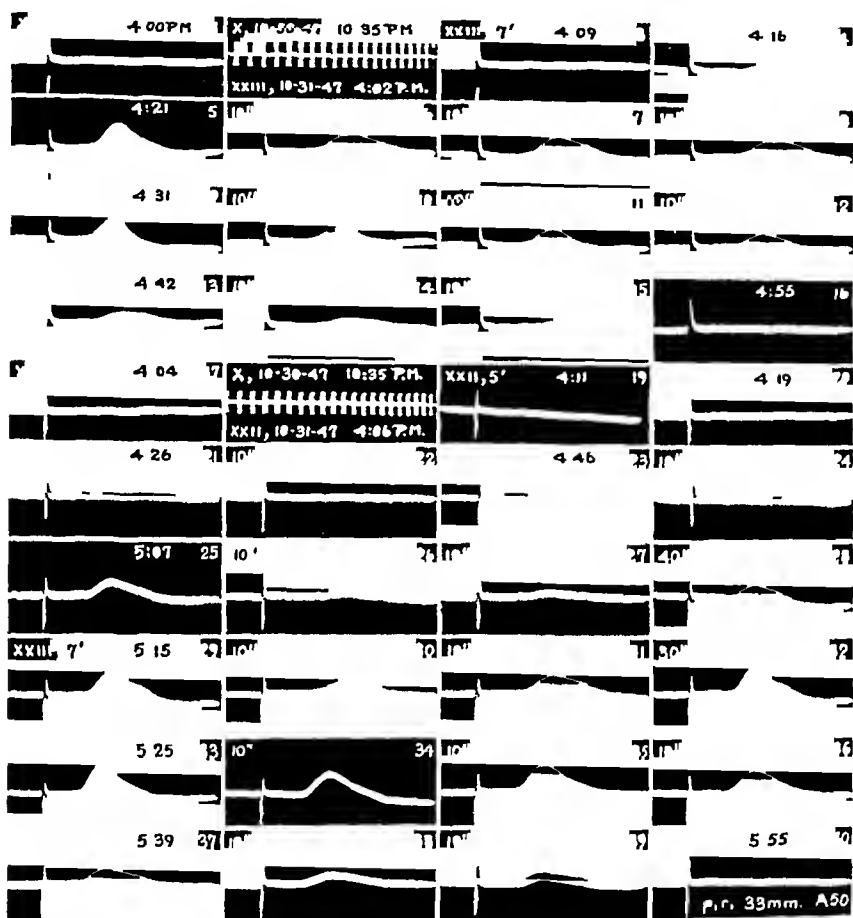


FIG 8 RESTORATION OF THE EXCITABILITY OF THE PERIPHERAL SEGMENT OF NERVES RENDERED INEXCITABLE IN 0.11 M DIETHANOL-DIMETHYL-AMMONIUM CHLORIDE

1, absence of conducted response in the peripheral segment, 3 to 16, restoration by tetra-n-butyl-ammonium ions

17, absence of conducted response in the peripheral segment, 22 to 28 restoration by tetra-n-propyl-ammonium ions, 29 to 40, effect of tetra-n-butyl-ammonium ions

of a specific property of the ethyl group. Thus, the initial working hypothesis had to be discarded and a new one had to be made in order to plan further experimental work

Let us consider the experimental facts that served to build up a new hypothesis. In the first place, the fact is significant that the number of quaternary ammonium ions that can substitute for sodium is great, indeed, among the ions listed in figures 1 and 6 no less than 15 are able to restore the ability to conduct impulses to Et fibers deprived of sodium. Since the nitrogen nucleus is the only thing that all those ions have in common, it must be concluded that the ability of a quaternary ammonium ion to substitute for sodium is not directly dependent upon a specific group being attached to nitrogen. The ability to substitute for sodium is a property that tetravalent nitrogen has when one of a number of combinations of groups with 2 or more carbon atoms are attached to it.

A further clue to the understanding of the problem is the fact that the restoring quaternary ammonium ions produce important changes in the properties of the nerve fibers. The experiment illustrated by figure 9 was designed with the purpose of demonstrating the fact in a clear manner.

The nerve was rendered inexcitable in 0.11 M diethanol-dimethyl-ammonium chloride. The central segment (cf fig 2, below, mp₂) was restored with Ringer's solution. Records 1 to 8 present the action potentials of impulses initiated at point p₁ and recorded at point m 1 hour after placing the central segment in contact with Ringer's solution. The spike in record 1 was produced by the fibers of the A group, the spikes in records 3 to 8 by fibers of slow conduction. The strength of the stimulus was increased progressively from record 2 to records 7 and 8. Under the conditions of the experiment, fibers of the C group do not begin to respond until the stimulus reaches strength 4. It will be noted that record 8 was obtained at a smaller sweep speed than records 2 to 7.

Records 13 to 23 present spikes recorded at point r₁ (cf fig 2, below) after restoration of the excitability of the peripheral segment by tetraethyl-ammonium ions. From the strength of the stimuli used for the individual records, it follows that the conducted responses in records 13, 14, 18 and 19 included spikes of fibers of the B group only, while the responses in the other records also included spikes of fibers of the C group. Thus, in spite of the great difference in the speed of conduction of B and C fibers in normal nerve, after restoration by tetraethyl-ammonium ions the B and the C fibers were conducting impulses

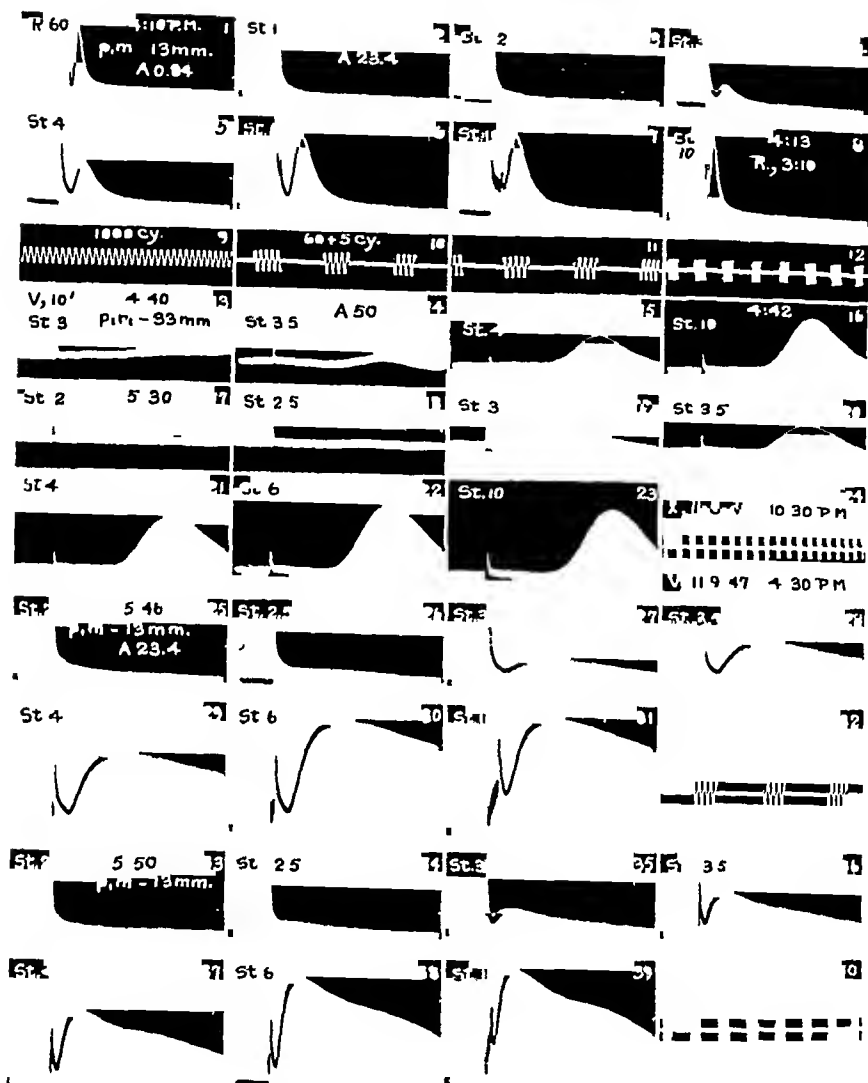


FIG 9 RESTORATION OF THE EXCITABILITY OF A NERVE RENDERED INEXCITABLE IN 0.11 M DIETHANOL-DIMETHYL-AMMONIUM CHLORIDE

1 to 8, conducted responses recorded at point m after restoration of the central segment by Ringer's solution. Time line 9 applies to record 1, time line 12 to record 8 and time lines 10, 11, to records 2 to 7. The strength of stimulation is indicated in arbitrary units in the upper left corner of the records.

13 to 23, conducted responses in the peripheral segment at 2 stages of the restoration by tetraethyl-ammonium ions. Time line 24 applies to records 13 to 23. 25 to 31 and 33 to 39, spikes of the fibers of slow conduction recorded again at point m after the action of tetraethyl-ammonium ions. Time line 32 applies to records 25 to 31, time line 40, to records 33 to 39.

at speeds of the same order of magnitude. Furthermore, the speeds of conduction were exceedingly small, since in segment mr_1 the fastest among the restored fibers were conducting at the rate of about 30–35 millimeters (*sic*) per second. In normal nerve the low threshold B fibers conduct at the rate of 3 m per second, and the low threshold C fibers at the rate of 0.8 m per second. Therefore, in nerve restored by tetraethyl-ammonium the speed of conduction is tremendously reduced, more so in the case of the B than in that of the C fibers.

Two other important changes in the properties of the nerve fibers are illustrated by records 25 to 39. These records present the spikes recorded at point m after the tetraethyl-ammonium ions had acted upon the mr_2 segment for 80 minutes. Since the segment p_1m had been kept in contact with Ringer's solution, the differences between records 2 to 8 and 25 to 39 may be regarded as a rather accurate measure of the changes in the properties of the nerve fibers that had been brought about by tetraethyl-ammonium ions. A comparison of, for example, records 8 and 39 readily shows that tetraethyl-ammonium ions had produced a tremendous lengthening of the duration of the spike of the action potential, as well as a marked increase in the height of the action potential. Therefore, no doubt can exist that the tetraethyl-ammonium ions had modified in an important manner those electrochemical reactions that underlie the production of the nerve impulse. In view of the experimental observations we may accept the proposition that tetraethyl-ammonium ions had been incorporated in the electrochemical mechanisms of the nerve fibers and had participated in a changed physiology of the fibers.

We may now build up a working hypothesis. Since the effect of restoring quaternary ammonium ions upon nerve is different from that of sodium, it is clear that the restoring ions do not substitute directly for sodium. We must believe rather that the restoring ions substitute for chemical species that the metabolic mechanisms of the nerve fibers synthesize by means of chemical reactions in which sodium takes part. In the absence of sodium the existing stores of those substances are progressively exhausted with the result that the nerve fibers become inexcitable. The restoring quaternary ammonium ions can substitute for the missing chemical species because they have a similar chemical structure.

This working hypothesis is, indeed, far reaching. It postulates that tetravalent (pentavalent) nitrogen plays an essential rôle in nerve physiology. The hypothesis also is very attractive, for the reason that it suggests a possible mechanism for the establishment of electric double layers in the nerve membrane.

Compounds containing trivalent nitrogen are weak bases, while the introduction of a 4th group, linked to nitrogen by covalency, results in an enormous increase in basicity and consequently in the appearance of electrovalent linkages. For this reason, a chemical reaction that results in the change of an amine into a quaternary ammonium base is a reaction that results in the creation of ions, i.e., in the appearance of charged particles of opposite signs, where previously electrically neutral compounds were present. Thus, we may assume that chemical reactions that result in a change of trivalent nitrogen into tetravalent nitrogen participate in the establishment of electric double layers in the nerve membrane.

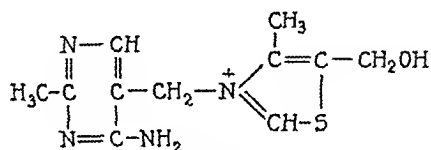
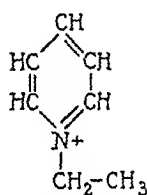
Whether or not the new working hypothesis will withstand the impact of experimental tests is a question that only future research may answer, but, however the question may be answered, the working hypothesis has already played the useful rôle of leading to profitable research along 2 different lines.

Since none of the restoring ions listed in figures 1 and 6 is a naturally occurring substance, it is clear that the working hypothesis would not rest upon solid ground until proof had been obtained that quaternary ammonium ions of the restoring type can be prepared from substances known to exist in nervous tissue. For this reason experiments were conducted with the quaternary ammonium ions listed in figures 10 and 11. One of the ions listed in figure 10, thiamine, is commercially available, the others were synthesized in the laboratory.

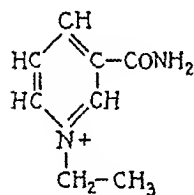
Thiamine (fig. 10, XXVI) is not able to substitute for sodium. If a nerve that has become inexcitable in a sodium-free medium is placed in contact with 0.11 M thiamine chloride, no nerve fiber regains its excitability, but if thereafter the nerve is placed in contact with 0.11 M tetraethyl-ammonium chloride the *Et* fibers rapidly become able to conduct impulses. Therefore, thiamine, if its action upon nerve is not prolonged beyond 1 hour, plays the rôle of an inert ion, it maintains the

osmotic equilibrium of the nerve fibers, but it cannot restore the excitability of nerve fibers deprived of sodium

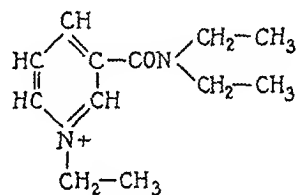
The quaternary ammonium ions derived from pyridine, niacinamide and coramine (fig 10, XXVII to XXIX) are inert ions, they do

XXVI
(Thiamine)

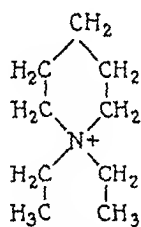
XXVII
(Ethyl-)
(pyridine)



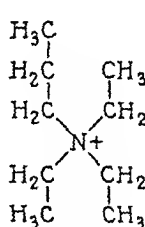
XXVIII
(Ethyl-
niacinamide)



XXIX
(Ethyl-coramine)



XXX	XVI
(Diethyl-piperidine)	(n propyl-triethyl ammonium)



XVI

FIG 10 QUATERNARY AMMONIUM IONS USED IN EXPERIMENTS DISCUSSED
IN THE TEXT

not restore the excitability of nerve fibers deprived of sodium, nor do they prevent later restoration by tetraethyl-ammonium

The quaternary ammonium ion derived from piperidine (fig 10, XXX) is active, as should be expected from the fact that it contains 2 ethyl groups. The ability of diethyl-piperidine to substitute for sodium is quite limited, as a matter of fact it is smaller than that of

dimethyl-diethylammonium (fig 1, III) On the other hand, if diethyl-piperidine is allowed to act upon the nerve for longer than 1 hour it causes an irreversible change in the Et fibers, which is not produced by dimethyl-diethyl-ammonium After diethyl-piperidine has acted upon the nerve longer than 1 hour the excitability of the Et fibers cannot be restored by tetraethyl-ammonium, and even sodium can effect only a slow recovery

For the purpose of comparison the formula of ion XVI (n-propyl-triethyl-ammonium) has been included in figure 10 The properties of n-propyl-triethyl-ammonium closely resemble those of tetraethyl-ammonium (cf fig 5, 25 to 36) One can think of ion XXX as having been derived from ion XVI by linking the n-propyl and 1 ethyl group to form a five-membered ring The introduction of this link results in an essential change in the biological properties of ion XVI, which is a further example of the dependence of the properties of quaternary ammonium ions upon the nature of the groups attached to nitrogen

Quaternary ammonium ions of the restoring type have been prepared from l (+) lysine and from histamine by submitting these substances to the action of boiling ethyl iodide in the presence of potassium hydroxide The resulting quaternary ammonium bases were isolated as ferrocyanates (see later)

In the case of lysine it is probable that 7 ethyl groups became attached to the molecule, 3 to each nitrogen and 1 to the carboxyl group, but since the resulting compound was treated with silver oxide to liberate the base and the base was then isolated as the ferrocyanate in the presence of 1.5 N sulphuric acid, it may be taken for granted that the ethyl ester was hydrolyzed and consequently the end of the lysine group acquired betaine structure The validity of the formula given in figure 11, XXXI will have to be confirmed by chemical analysis of the base that was used for the experiment illustrated by figure 12, for the purpose of the present discussion, however, it is sufficient to know that lysine can be ethylated to become a strong base that is precipitated from acid solution by potassium ferrocyanide and that is able to restore the excitability of Et fibers deprived of sodium

The base resulting from ethylation of histamine has not been analyzed yet It probably is one of the 2 bases listed in figure 11, XXXII,

record 25 indicates that ethylated lysine had restored the excitability of at least the majority, and probably of the totality of Et fibers

For the purposes of comparison, the formula of ion XIX (n-hexyl-triethyl-ammonium) has been included in figure 11. As already mentioned, the properties of ion XIX are quite different from those of tetraethyl-ammonium, therefore, the fact is worthy of emphasis that the properties of tetraethyl-ammonium are obtained again by replacing the 2 last carbons of the n-hexyl group by the betaine structure that appears in figure 12, XXXI

The effect of ethylated histamine upon frog nerve deprived of sodium is illustrated by figure 13. The nerve was allowed to become inexcitable in 0.11 M diethanol-dimethyl-ammonium chloride. The restoration was effected with a 0.11 M solution of the chloride of ethylated histamine (titrated as the chloride of a monoacid base). No conducted response was observed 11 minutes after the solution had been placed in contact with the nerve (record 4), but 11 minutes later a large number of Et fibers were able to conduct impulses (records 5 to 8). The height of the conducted response increased progressively with advancing time (records 9 to 24), in part the increase was referable to an increase in the number of responding fibers, but after the action of ethylated histamine had lasted for an hour the further increase in the spike height undoubtedly was due to an increase in the height of the individual fiber spikes. In support of this conclusion there are 2 facts: (1) the speed of conduction decreased progressively (cf records 5, 9, 13, 17, 21), and (2) the spike was followed by a very large negative after-potential (records 25 to 27). Also, the L fraction of the membrane potential of the A fibers underwent a great increase. Ethylated histamine, therefore, was producing those changes in the properties of the nerve fibers which are so characteristic of the action of tetraethyl-ammonium, the only difference was that the changes produced by ethylated histamine were even greater than those which are produced by tetraethyl-ammonium.

The nerve was finally placed in contact with Ringer's solution, and, as was expected, the speed of conduction underwent a marked increase (cf records 29 to 31 with 21 to 24). The spike height also increased, which probably was referable to a diminution of the effect of temporal dispersion of the individual fiber spikes. With advancing

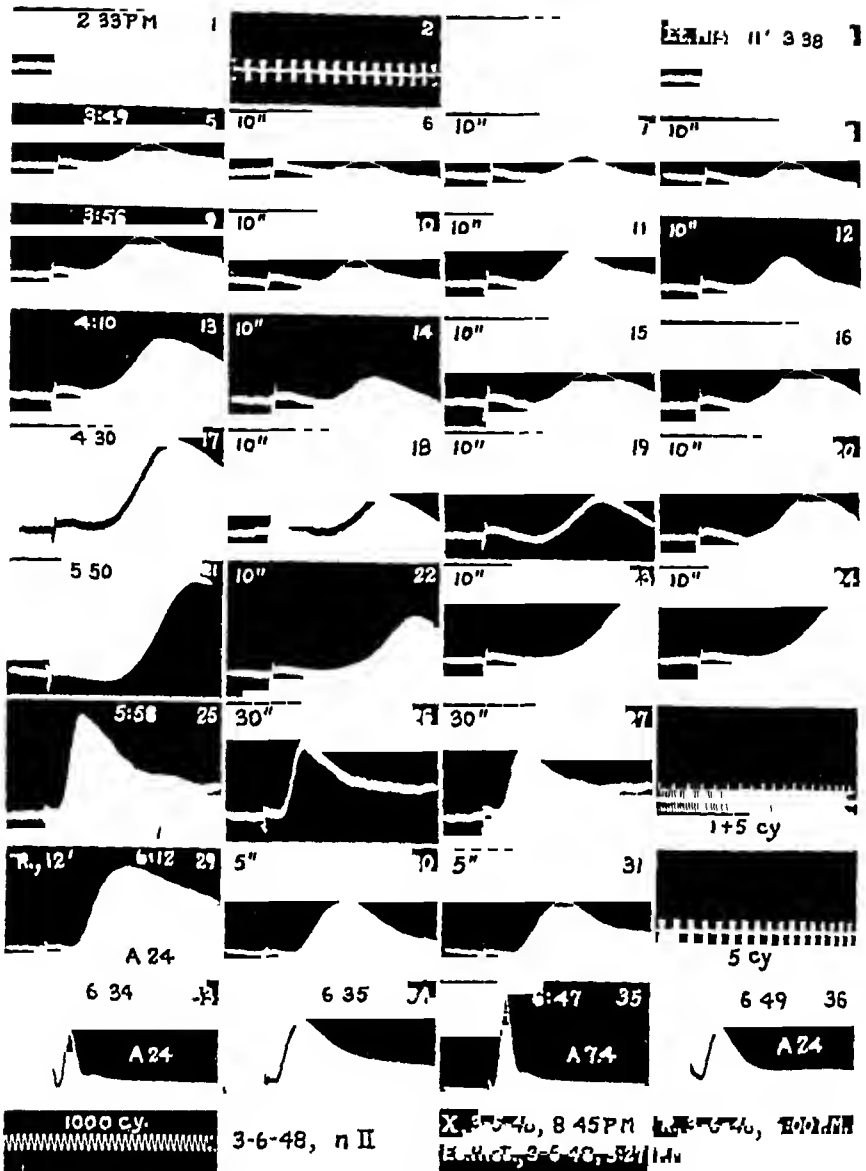


FIG 13 RESTORATION OF THE EXCITABILITY OF THE PERIPHERAL SEGMENT OF A NERVE RENDERED INEXCITABLE IN 0.11 M DIETHANOL-DIMETHYL-AMMONIUM CHLORIDE

1, absence of response in the peripheral segment of the nerve, 4 to 27 restoration of the excitability of Et fibers by ethylated histamine. The amplification was constant (A 50). Records 1 to 24 were obtained with the sweep speed of record 2, records 25 to 27 with that of record 28. 29 to 36, restoration by Ringer's solution. Time line 32 applies to records 29 to 31, 34 and 36, the time line below record 33 to records 33 and 35.

time, the height of the Et spike decreased progressively, as is shown by records 34 and 36, tending to reach the height that it has in normal nerve. Obviously, the changes in the properties of the nerve fibers produced by ethylated histamine disappeared progressively in the presence of sodium. Records 33 and 35 illustrate 2 stages of the recoveries of the fibers of fast conduction.

Emphasis may be placed upon the similarity that exists between the effects upon nerve of ethylated histamine and of tetraethyl-ammonium. Also worthy of emphasis is the fact that beta-phenylethyl-triethyl-ammonium (fig 6, XXI) and ethylated histamine have different properties. The ability of beta-phenylethyl-triethyl-ammonium to restore Et fibers deprived of sodium is quite limited, furthermore, beta-phenylethyl-triethyl-ammonium after it has acted upon the nerve for about one hour makes the restored fibers inexcitable and prevents restoration by tetraethyl-ammonium. Ethylated histamine is a very powerful restoring agent that maintains the excitability of the restored fibers for at least several hours.

There can be no doubt that the preparation of quaternary ammonium ions of the restoring type from naturally occurring substances makes the working hypothesis plausible. Indeed, the fact that a restoring ion can be prepared from lysine suggests this possibility, that the basic end groups of protein molecules could be places where amino-nitrogen is converted into tetravalent nitrogen and conversely.

Let us consider now the second research that has been directed by the working hypothesis, namely the search for quaternary ammonium ions of the restoring type in nervous tissue. Obviously, if quaternary ammonium ions with properties resembling those of the restoring ions should participate in nerve function, the direct manner of demonstrating their existence would be to isolate them from nervous tissue. The isolation has been accomplished.

A remarkable property of the restoring ions has made the isolation a relatively easy task. As was discovered by E. Fischer (1878), potassium ferrocyanide precipitates quaternary ammonium bases from strongly acid solution. It has appeared, however, that the reaction is not universal, since there are quaternary ammonium bases that are not precipitated by potassium ferrocyanide. Remarkable enough, choline and acetylcholine are not precipitated, even not from concentrated solutions, while practically all the quaternary ammonium ions

of the restoring type are precipitated from dilute solutions. For this reason, it was expected that if quaternary ammonium ions of the restoring type were present in nervous tissue it would be possible to isolate them in the form of ferrocyanates.

Ox brains (5 kg) were minced in 5 l of distilled water and concentrated hydrochloric acid was added to make the mixture approximately 0.1 N in HCl. The mixture was boiled under a reflux condenser for 4 hours. The filtrate was submitted to the various steps of the Kossel-Kutscher procedure for isolation of the basic amino acids (cf Winterstein, '33), except that the precipitation with silver sulphate was effected at pH 9. After the removal of barium and silver, the filtrate was concentrated on the water bath to a thin syrup (approximately 200 ml), and after addition of sulphuric acid to pH 0.5 small volumes of a saturated solution of potassium ferrocyanide were added until no further precipitation occurred. The precipitate was suspended in water, the ferrocyanide was removed with copper sulphate, the excess copper and the sulphate with barium hydroxide, and the slight excess of barium with carbon dioxide. Hydrochloric acid was added to pH 5 and the solution was boiled for a few minutes. The solution was evaporated on the water bath and the residue was extracted with a small volume of boiling methyl alcohol. The methyl alcohol solution was evaporated and the residue dried in vacuum at 80°C. The residue consisted in part of a crystalline powder, probably white, and in part of an amorphous greenish-yellow material. Both substances proved to be very soluble in water, readily soluble in methyl alcohol and almost insoluble in cold ethyl alcohol. No attempt was made to separate the two substances. Finally, the residue was dissolved in a small amount of water.

The solution proved to contain the chloride of a base that is strong, since the solution was practically neutral (pH 6.7). For its use in experiments on nerve the volume of the solution was adjusted so that the concentration of Cl^- ions became 0.1 N. The volume of the solution was then 21 ml, thus, on the assumption that the active substance is a monoacid base, the yield of the extraction from 5 kg of brains was approximately 2 millimoles. Appropriate tests showed that the solution contained only traces of potassium and of sodium, i.e., that these 2 ions were present only in amounts many times smaller than those which still have demonstrable effect upon frog nerve.

The effect of the extract upon Et fibers deprived of sodium is illustrated by figure 14, that reproduces records obtained in 2 different experiments (1 to 16 and 17 to 31). In the 2 cases the nerves were allowed to become inexcitable in 0.11 M diethanol-dimethyl-ammo-

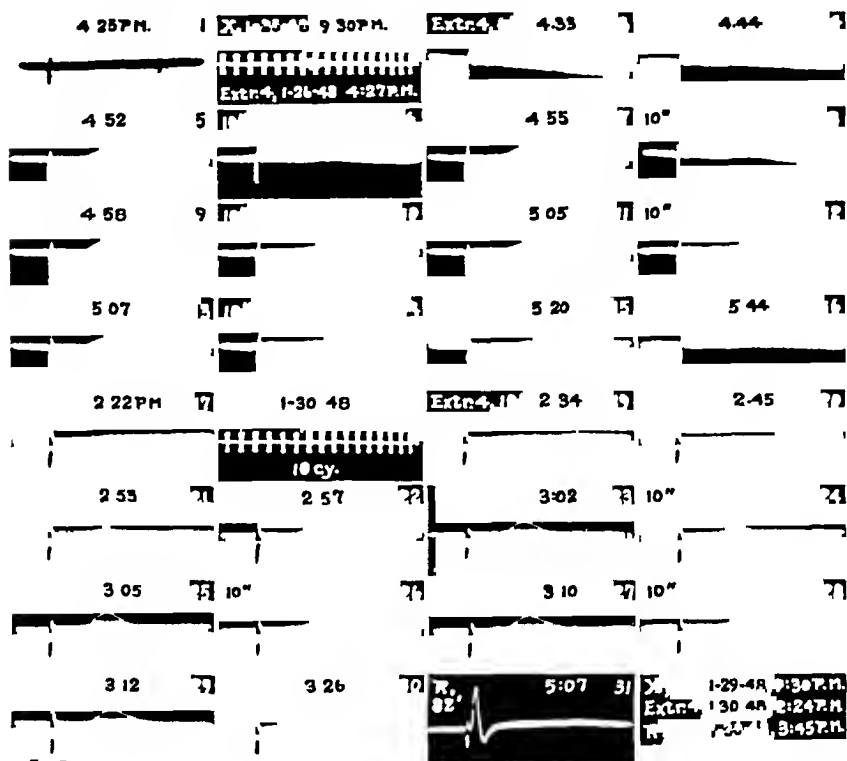


FIG. 14 RESTORATION OF THE EXCITABILITY OF NERVES RENDERED INEXCITABLE IN 0.11 M DIETHANOL-DIMETHYL-AMMONIUM CHLORIDE

1 and 17, absence of conducted response in the peripheral segments of the nerves, 5 to 16 and 19 to 30, restoration of the excitability of Et fibers by the base contained in brain extract 4, 31, spike of the fibers of slow conduction after restoration by Ringer's solution. The sweep speed and the amplification ($\times 50$) were constant.

nium chloride (records 1 and 17). The restoration of the ability of Et fibers to conduct impulses did not begin as rapidly as it usually does when 0.11 M tetraethyl-ammonium chloride is used, but in both cases a number of Et fibers were found to be able to conduct impulses 20 minutes after the brain extract had been placed in contact with the nerve (records 5, 6, 21). In both cases the conducted response in-

creased with advancing time (records 5 to 9, 21 to 25), later, the response decreased in size progressively (records 11 to 16, 27 to 30), a phenomenon that is not observed with tetraethyl-ammonium, but is produced by other restoring ions (cf figs 7 and 8) It will be noted that the speed of conduction of the restored fibers was exceedingly low, which is one of the most pronounced features of the action upon nerve of restoring quaternary ammonium ions

In the second experiment the peripheral segment of the nerve was treated with Ringer's solution after it had been in contact with the brain extract for one hour The speed of conduction and the spike height were observed to increase progressively, record 31 presents the fully recovered spike of the fibers of slow conduction A comparison of records 25 and 31 shows that the extract had restored the excitability of an important number of Et fibers

In view of these results there can be no doubt that the extract contains a substance that has properties similar to those of quaternary ammonium ions of the restoring type In one important respect, however, the naturally occurring substance differs from the restoring ions listed in figures 1, 6, 10 and 11 The brain extract restores to fibers of fast conduction (A fibers) the ability to produce impulses

Figure 15 reproduces records of electrotonic potentials in the peripheral segments of 2 nerves The potentials were produced by rectangular pulses of current and were recorded at 6 mm from the polarizing electrode Records 1 to 4 define the state of the fibers of fast conduction of the 2 nerves before the application of the restoring solutions In one case (records 5 to 8) the restoration was effected with ethylated lysine (cf fig 12), and in the other case (records 9 to 24), with the brain extract (cf fig 14, 1 to 16)

Ethylated lysine did not produce any readily detectable change in the recorded potentials, in particular, the rounded corner of the deflection produced by the break of the anodal current (records 5 to 8) was a proof that the A fibers failed to produce impulses in response to the break of the current The brain extract, however, caused important changes In the first place, attention will be called upon the fact that the corners of the deflections in records 9 to 12 are quite sharp, the fact indicates that certain features of the electrotonic potential of normal nerve had been restored by the extract On the other hand,

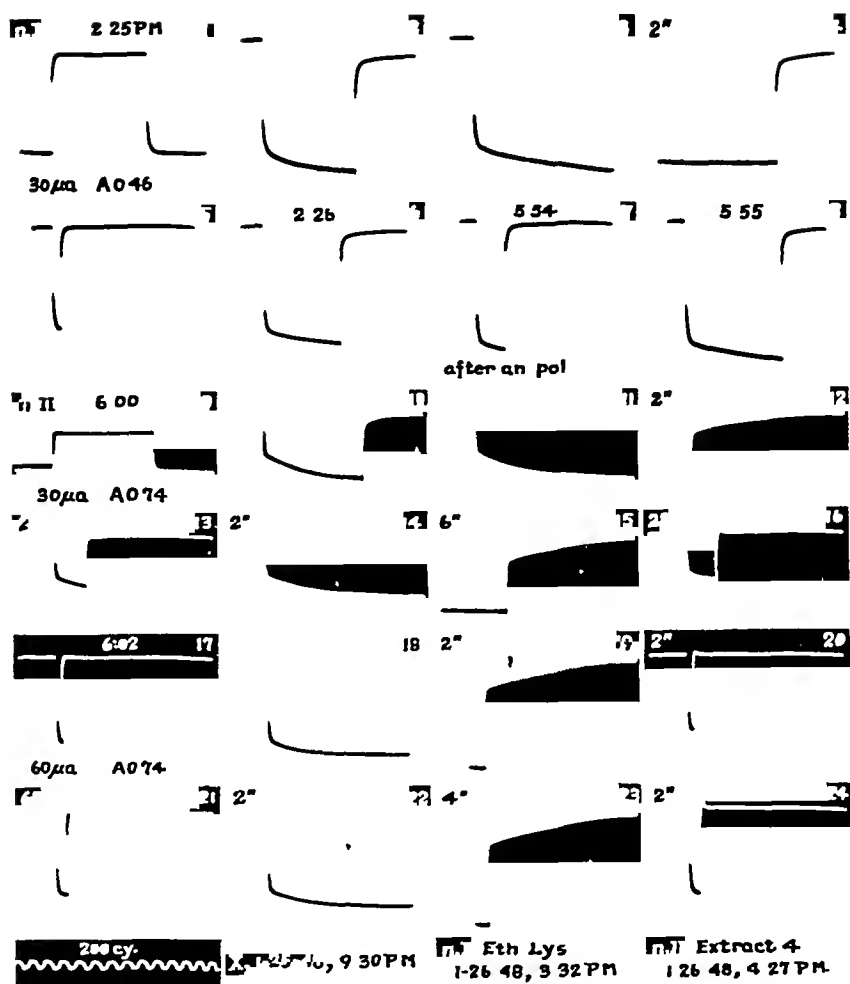


FIG 15 COMPARISON OF THE EFFECTS OF ETHYLATED LYSINE AND OF BRAIN EXTRACT 4 UPON THE ELECTROTONIC POTENTIALS PRODUCED IN THE PERIPHERAL SEGMENT OF THE NERVE BY APPLIED CURRENTS

1 to 4, electrotonic potentials recorded before the application of the test solutions, 5 to 8, effect of ethylated lysine, 9 to 24, effect of brain extract 4. Note the spikes of A fibers superposed upon the decay of the anelectrotonus in records 16, 20, 21 and 24.

impulses were produced by A fibers in response to the break of the anodal current, provided only that the state of the nerve had been improved by a preceding period of anodal polarization. In the case of

records 13 and 17 few fibers, if any, produced impulses, but in the case of records 16, 20, 21 and 24 there is no doubt that a significant number of A fibers responded to the break of the current, since unmistakable spikes appear superposed upon the deflection produced by the decay of the anelectrotonus. Clearly, after the brain extract had acted upon the nerve the periods of anodal polarization that were used to obtain records 14, 15, 18, 19 and 22, 23 improved the state of the nerve fibers to the extent that nerve impulses could be produced (cf Lorente de Nó, '47, Chapter XIII)

Identification of the active substance by chemical analysis has not been done yet, consequently, the nature of the substance is still unknown. As a matter of fact, in view of the results of experiments done with the use of another brain extract it seems plausible to believe that two substances with somewhat different properties are present in the extracts. But, however this may ultimately prove to be, there can be no doubt that at least one substance can be extracted from the ox brain that in all probability is a quaternary ammonium base and is different from choline, acetylcholine and thiamine, both in chemical and in biological properties. The substance extracted from the ox brain restores the excitability of frog nerve fibers deprived of sodium. Therefore, it is logical to conclude that the substance participates in the accomplishment of nerve function, but only future research may disclose the intimate details of the mode of action of the naturally occurring substance or substances and of the mode of action of the restoring ions listed in figures 1, 6, 10 and 11.

The problem presented by the differences in the actions of quaternary ammonium ions upon frog nerve is only a new aspect of an old problem, that of the relationship of chemical structure to biological action. In a masterly lecture Sir Henry Dale ('20), making specific reference to the then known differences between the pharmacological actions of tetramethyl-ammonium and tetraethyl-ammonium (cf Burn and Dale, '15), used these words: "A few years ago, in discussing this curious contrast between the methyl and ethyl ammonium bases, I ventured to say that it was as mysterious as the physiological contrast between sodium and potassium." No more graphic statement of the nature of the problem could be made today.

That in its action upon nerve tetraethyl-ammonium resembles

sodium need not be mentioned again. The action of tetramethylammonium does not resemble that of potassium, but a number of quaternary ammonium ions exert a depolarizing action upon nerve, so that, in this respect, their action resembles that of potassium. Remarkably enough, in spite of differences in the abilities of the ions to substitute for sodium in experiments of short duration, 0.11 M solutions of the chlorides of all the ions listed in diagonal 4,d of figure 1 are powerful depolarizing agents, to the extent that 3 of them cause disintegration of the nerve membrane even faster than 0.11 M potassium chloride.

The question as to why the properties of tetravalent nitrogen may vary so widely, depending upon the nature of the groups attached to it, undoubtedly will address serious challenges to theoretical chemists. Probably, the action of many additional ions will have to be investigated before the number of series of ions with gradual structural differences, that has been submitted to experimental analysis, becomes sufficient for a successful theoretical study.

There still is one question that should be discussed in this report, the action of acetylcholine upon peripheral nerve fibers. The formula of acetylcholine has been included in figure 6 within a frame in order to emphasize the fact that acetylcholine does not belong to the class of restoring ions. On the contrary, acetylcholine is one of the inert ions, provided only that its enzymatic hydrolysis be prevented by means of a cholinesterase inhibitor. Certain experimental observations may be recalled here (cf. Lorente de Nó, '47, section IV, 7).

If minute amounts of an esterase inhibitor, eserine, prostigmine or di-isopropyl-fluorophosphate, are added to a solution of acetylcholine, the solution proves to have no direct action upon the nerve fibers, even when the solution is isosmotic with Ringer's solution. To be sure, an isosmotic solution of acetylcholine ultimately renders the nerve fibers inexcitable, because it is a sodium-free medium, but if the solution contains sodium ions at the concentration 0.022 M the nerve fibers remain excitable for at least 24 hours in the presence of acetylcholine (1.6 per cent), provided only that, be it repeated, the solution contains a cholinesterase inhibitor at concentrations of the order of magnitude of those which are sufficient to inhibit cholinesterase in *in vitro* experiments. Therefore it must be concluded that acetylcholine has no demonstrable

action upon the mechanism that underlies the production of the nerve impulse, nor upon the mechanism that maintains the resting membrane potential. In so far as peripheral nerve is concerned, and as far as present-day methods go, acetylcholine is an inert substance.

An entirely different situation prevails if the experiments are done in the absence of a cholinesterase inhibitor. It is true that acetylcholine, at concentrations up to the indeed high concentration of 0.2–0.3 percent, fails to have a demonstrable action upon peripheral nerve, in the absence as well as in the presence of an esterase inhibitor, at higher concentrations, however, acetylcholine produces a progressive depolarization of the nerve fibers, and soon the nerve fibers become inexcitable. Obviously, uninhibited cholinesterase splits acetylcholine to yield one or more substances that exert a deleterious action upon nerve. One of the products of the hydrolysis of acetylcholine is choline (fig. 1, VI), which is an inert ion, therefore, the active substance must be the other product of the hydrolysis, acetic acid. As a matter of fact, appropriate experiments have demonstrated that, in contrast with the effect of certain other organic acids, acetic acid exerts a deleterious action upon nerve (Lorente de Nó, '47, section III, 7). Thus, there can be no doubt that both acetylcholine and the cholinesterase inhibitors (eserine, prostigmine and di-isopropyl-fluorophosphate) penetrate into the nerve fibers, at least as far as is necessary to reach the cholinesterase. On the other hand, the fact that the ethyl homologue of acetylcholine is a restoring ion (fig. 7, 5 to 12) leaves no theoretical reason to support the assumption that acetylcholine would not penetrate into the nerve fibers. Thus, the only permissible statement is that, in regard to peripheral nerve, acetylcholine is an inert substance.

The lack of demonstrable action of acetylcholine itself upon peripheral nerve stands in sharpest contrast with the exceedingly powerful action that minute concentrations of acetylcholine have upon the heart and upon the synaptic junctions, among others of muscle and of sympathetic ganglia. This difference between the action of acetylcholine upon nerve and upon synaptic junctions is one of the fundamental facts of neurophysiology. The fact is an important part of the foundation upon which Loewi ('33) and Dale ('37) based their concept of synaptic transmission. Later work has only increased the importance of the fact. Therefore, in concluding, I may state that

the difference between the actions of acetylcholine upon nerve and upon the synaptic junctions must be regarded, now more than ever, as one of the basic facts of neurophysiology

SUMMARY

An analysis has been made of the effect upon the excitability of frog nerve fibers deprived of sodium of 33 quaternary ammonium ions and of a base extracted from the ox brain, which in all probability also is a quaternary ammonium ion

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DISCUSSION

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This work on quaternary ammonium compounds opens up valuable new approaches to nerve physiology It will certainly stimulate profitable new experiments along diverse lines

In previous studies Lorente de Nó established that Na^+ ions are not essential for the maintenance of the nerve membrane potential

Further, a nerve depolarized by KCl may regain its polarized state in the absence of Na^+ ions, e.g. while surrounded by 2.5 per cent choline chloride in distilled water or acetylcholine in similar concentrations. Na^+ ions, however, appeared to play an essential rôle in the production of propagated responses.

The experiments reported in Dr Lorente's paper are a logical continuation and extension of those studies. They also represent an important step in the direction of correlating structural composition of pharmacological substances, with physiological function. Similar attempts have been notoriously unsuccessful in the past and have prompted A. J. Clark to a statement which read somewhat as follows: "Sufficient work on the correlation of structure and action has been done to make clear the extent of our ignorance in this field."

From these or previous studies, it is not known how long it takes to remove sodium from nerve, if it is surrounded by solutions lacking this ion. It would therefore be interesting to correlate the actual sodium content with the ability of nerve to conduct impulses. Should all appreciable amounts of sodium disappear within several hours, then it would demonstrate that (1) Na^+ ions are not indispensable for conduction. It would also further support the assumption that (2) stores of essential "products" or metabolites, which depend for their synthesis on Na^+ ions, get depleted during those remaining hours of conduction in the absence of sodium. In this connection, the following findings of Tobias are of interest, namely that muscles soaked in distilled water for 5-48 hours lose all their potassium, but still retain some sodium together with an appreciable membrane potential of 10-30 mV. Further, sodium may be practically absent in certain silkworm pupae while conduction apparently exists.

Dr Lorente's experiments do not favour a view that in medullated nerve conduction a potassium-sodium exchange takes place during nerve impulses.

That quaternary ammonium ions enter into the mechanism of nerve impulse production is clear from the profound modifications which they effect on the conduction mechanism. As Dr Lorente points out, however, this does not prove that they normally take part in those processes. It need not be assumed that a great variety of ions, of different composition, could not act in a similar manner. This aspect of the

problem is well on the way to solution through the studies using brain extracts

One of the most interesting suggestions emerging from this study is the possible mode of creation of electric double layers by compounds which apparently occur normally in nervous tissue. The transformation of trivalent nitrogen compounds into tetravalent ones during the recovery phase, e.g. of depolarized nerve, would adduce strong evidence in support of such a mechanism. Studies with heavy nitrogen may be most useful in this approach.

The selective effect of tetraethyl-ammonium compounds on nerves of different diameter and conduction velocities supplies us with a valuable new tool of investigation of the properties of these fibers. Conduction velocities as low as 30 mm/sec have never been observed before in vertebrate nerve. Such drastic changes are of interest, since the mode of propagation must be profoundly altered.

The failure of tetraethyl-ammonium to restore the large diameter A fibers while brain extracts will be effective is an indication that the latter may eventually yield a series of interesting compounds.

The problem of penetration of the diverse compounds into the nerve fibers has not been taken up in this short paper. Injection of radioactive material, or soaking of nerves in it, would undoubtedly help in determining the extent to which the quaternary ammonium compounds participate in nervous activity. All these possibilities have been opened up by Dr Lorente's recent work.

REFERENCE

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III ACETYLCHOLINE AS A PHARMACOLOGICAL AGENT

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Acetylcholine has been assigned the important physiological rôle of chemical mediator of parasympathetic postganglionic, autonomic pre-ganglionic (synaptic) and motor nerve impulses. The theory of this chemical mediation is based partly on the recovery of acetylcholine in the venous effluent from the stimulated effector structures and partly on the similarity of physiological response to nerve stimulation and to acetylcholine injection. Nachmansohn and Rothenberg (34) hold that the theory of acetylcholine being involved in the chemical mediation of nerve impulse to the effectors is based essentially on pharmacological evidence which does not permit conclusions as to the site of the physiological action of acetylcholine. It is well known that these investigators assign to acetylcholine a physiological rôle in nerve conduction and state that the release and the removal of this substance are essential factors in conduction. These authors believe that the theory of acetylcholine as a transmitter is deduced purely from the pharmacological action of acetylcholine, and do not appear to attach significance to the recovery of acetylcholine in the venous effluent from stimulated, cholinergically innervated structures (in contrast to adrenergically innervated structures).

The protagonists of the transmitter theory maintain that sufficient evidence exists for the rôle of acetylcholine as a chemical mediator, even if the pharmacological action of acetylcholine and the physiological effects of nerve stimulation should prove to be not exactly identical. They have recently attributed additional rôles to acetylcholine in nervous activity, claiming that it is the neurohormone of the central nervous system. According to Gray (14), intra-arterial injection of acetylcholine into the skin produces in cats and dogs discharge of fast sensory nerve impulses lasting for several seconds. He suggests that sensory endings, like the central ends of postganglionic fibers and motor end-plates, may be specialized to facilitate the starting of propagated responses.

Whether one adheres to the Loewi-Dale theory of chemical mediation or to the Nachmansohn theory of conduction, the fact remains, and is admitted by both, that acetylcholine has an important physiological rôle in the activity of the nervous system, and has clearly defined and predictable pharmacological actions. Therefore, it is imperative to assemble and critically examine the major experimental evidence relative to the pharmacological action of injected acetylcholine.

For the sake of brevity, it is impossible to review many important effects of acetylcholine, such as its influence on the activation of marine eggs, on the light-dark-adapted retina, on the cord activity of earthworms, or on the genital organs of male animals in conjunction with the effect of sex hormones.

Since acetylcholine is rapidly destroyed by the cholinesterase system, the most reliable results are obtained when the drug is injected directly into the blood vessels. Pharmacologists, like physiologists, are interested in the cholinesterase problem, particularly in cholinesterase inhibitors (reversible and irreversible), because they find that not only the effects of cholinergic nerve stimulation, but also those of injected acetylcholine are prolonged, potentiated or otherwise modified by anti-cholinesterases. It may be convenient to distinguish between two types of cholinesterases: (a) "*specific cholinesterase*,"¹ the enzyme present in the effector organ and responsible for terminating acetylcholine effect upon any specific stimulated structure, (b) "*transport cholinesterase*," the enzyme with which the drug comes in contact before reaching the responsive structures. It is also responsible for the destruction of any acetylcholine that might escape hydrolysis by the specific cholinesterase. The term "*essential cholinesterase*" should be reserved for those fractions of specific and transport cholinesterases that limit the magnitude and persistence of acetylcholine effect on any given structure, e.g. sum total of cholinesterase concerned with vaso-depressor effects of acetylcholine.

This review of acetylcholine effects on the various systems differentiates, whenever possible, between the muscarinic and nicotinic effects of this drug.

¹ It is unfortunate that true cholinesterase is occasionally referred to as "specific cholinesterase."

A ALIMENTARY TRACT

Intravenous or intra-arterial injection of acetylcholine increases the tone, the rhythmic contractions, and the peristaltic activity of the gastrointestinal tract. Localized contraction rings and violent spasm, followed by defecation or diarrhea, may also result from acetylcholine injection. Molitor (33) found that acetylcholine, in concentrations of 0.001 mg percent, increases the amplitude and frequency of the movements of isolated rabbit intestine, and also in non-anesthetized dogs it produces vomiting and defecation. The *minimum effective cathartic* dose of acetylcholine in dogs is, according to Molitor, 0.8 mg/kg upon subcutaneous, and 40 mg/kg upon oral administration. We found that in non-anesthetized dogs (six animals) doses of 1 mg/kg or less failed to produce defecation (intravenous administration). Intravenous doses of 2.5 mg/kg or more were necessary to produce nausea and vomiting (probably central effects). In man, Weiss and Ellis (43) found that a 2 percent solution of acetylcholine injected intravenously at a rate of from 0.02 to 0.14 Gm per minute produced nausea and vomiting. Carmichael and Fraser (8) did not observe nausea and vomiting in similar experiments (different injection rates?).

Atropine abolishes the pendulum and peristaltic movements and lowers the tonus. Only the administration of optimum amounts of anticholinesterases (0.1 to 1.0 mg/kg of hexaethyltetraphosphate ("HEPT"), or tetraethylpyrophosphate ("TEPP")), can restore the tone and establish rhythmic movements in atropinized animals. In most mammals premedicated with atropine and cholinesterase inhibitors, massive doses of acetylcholine may only increase the tone without increasing the rhythmic or peristaltic activity. Close intra-arterial injection of "TEPP" produces marked intestinal stimulation in atropinized animals.

Experiments on atropinized animals reveal that there is no fundamental difference between the effects of peripheral vagus stimulation and of acetylcholine injection on the gastric and duodenal tonus and motility. In twelve dogs the following results were obtained

- a Atropine administered intravenously in doses from 1 to 10 mg/kg uniformly produced a lowering of the tone and stoppage of motility. Duodenal motility was recorded by a tambour connected with a balloon inserted through the stomach into the duodenum.



FIG 1 Dog, 8.6 kg, 35 mg of sodium pentobarbital, 0.1 mg of atropine sulfate and 0.5 mg of prostigmine per kilogram by vein A, line indicating injection of drugs B, time, 2 seconds, base line representing zero mm of Hg pressure C, blood pressure tracing from the common carotid artery D, tracing of duodenal motility E, 0.05 mg of acetylcholine chloride F, 0.05 mg of acetyl-beta-methyl-choline chloride G, 0.1 mg of acetylcholine chloride H, 0.3 mg of acetylcholine chloride I, following 30 mg of nicotine salicylate, 0.1 mg of acetylcholine chloride

- b Simultaneous faradic stimulation of both vagi initiated duodenal contractions in only two of twelve dogs, and in only six of over one hundred faradic stimulations
- c Large doses of acetylcholine (2.5 to 5.0 mg/kg) increased the tonus in eight of twelve dogs
- d When these animals were given large amounts of "HETP" or "TEPP", vagus stimulation and acetylcholine injection often produced both increase in tonus and motility
- e Acetylcholine in the presence of 0.1 mg/kg of atropine and of 0.5 mg/kg of physostigmine or neostigmine produced pressor (nicotinic) effects preceded by a fall of blood pressure (muscarinic effects). Under these conditions, vasodepression was accompanied by stimulation of intestinal activity, while the marked pressor effect was accompanied by unmistakable intestinal relaxation (epinephrine-like effect) (Fig. 1)
- f Large amounts of nicotine (5 to 10 mg/kg) abolished both the pressor and the intestinal inhibiting effects of acetylcholine

The intestinal relaxation must be looked upon as a true nicotinic effect of acetylcholine. It was also observed by Hoffmann et al (15), who demonstrated that the perfusion of isolated mammalian hearts with acetylcholine solutions, producing positive ino- and chronotropic effects (nicotinic action), results in the perfusate acquiring the property of relaxing the rectal cecum of the fowl and the small intestine of the rabbit.

The observations that in some atropinized animals large doses of acetylcholine increased intestinal tone and motility, while in others produced pure relaxation, are not contradictory when it is realized that the former represent a "breaking through" of the muscarinic effect commonly seen in other systems as well.

Salivary secretion is readily produced by intravenous injection of acetylcholine both in animals and in man. Premedication with sub-minimal doses of anticholinesterases makes the flow more copious and lasting.

Wilkinson (45) investigated the effect of subcutaneous doses of acetylcholine (0.1 to 0.2 Gm, total) on gastric secretion in normal and ill human subjects. In the majority of normal cases he found an increase of the free and total gastric acidity. In pathological conditions (pernicious anemia, secondary anemia, ulcers with acid hypersecretion) variable results were obtained. In mucous colitis, acetylcholine failed to increase free hydrochloric acid, but produced an

increase in the total gastric chlorides Atropine prevented or abruptly terminated the effects of acetylcholine Necheles, Motel and Kosse (35) compared the effects of acetylcholine, methacholine, and neostigmine on gastric secretion, and found that acetylcholine was the weakest stimulant among the drugs employed Subcutaneous doses (0.1 to 0.4 Gm, total) caused a distinct increase in volume of gastric juice, and of acid and pepsin secretion of the Heidenhain pouch in dogs The maximum effect was obtained 30 minutes after injection and lasted for over an hour Neostigmine produced similar results and appeared to enhance the effects of acetylcholine

Stavraky (40) injected acetylcholine into the gastrosplenic artery and reported an increased secretion of alkaline gastric juice, at times as high as pH 8.9 This secretion apparently originates from the inner third of the glands of the body of the stomach along the greater curvature and the chief cells of the neck Stavraky (40) states that acetylcholine is not a powerful stimulant for hydrochloric acid, and a preexisting secretory activity in the stomach is needed for its acid-stimulating effect In excessive doses, or by intra-arterial injection (particularly in a quiescent stomach), acetylcholine induces alkaline secretion It may be conjectured that the acid-stimulating effect is a muscarinic action, while the alkaline secretion is possibly nicotinic

Villaret and Justin-Besancon (42) observed in dogs, following intravenous injection of acetylcholine, an abundant secretion of pancreatic juice rich in lipase and amylase

B HEART AND PERIPHERAL CIRCULATION

Acetylcholine in small doses produces a fall of blood pressure without cardiac inhibition, indeed, a reflex acceleration of the heart more often occurs Larger doses (0.005 or 0.01 mg/kg or higher) produce cardiac slowing and even asystole In animals in which the negative chronotropic (vagal) effect has been abolished by atropine or by methylene blue² (McDowall, 28), acetylcholine in doses of 0.25 mg/kg or more produces a nicotinic type of cardiac acceleration

² In this laboratory we were unable to demonstrate in anesthetized dogs an atropine-like effect of methylene blue in doses from 2.5 to 60.0 mg/kg given by vein Rather the methylene blue appeared to increase the vasodepressor and negative chronotropic effects of acetylcholine

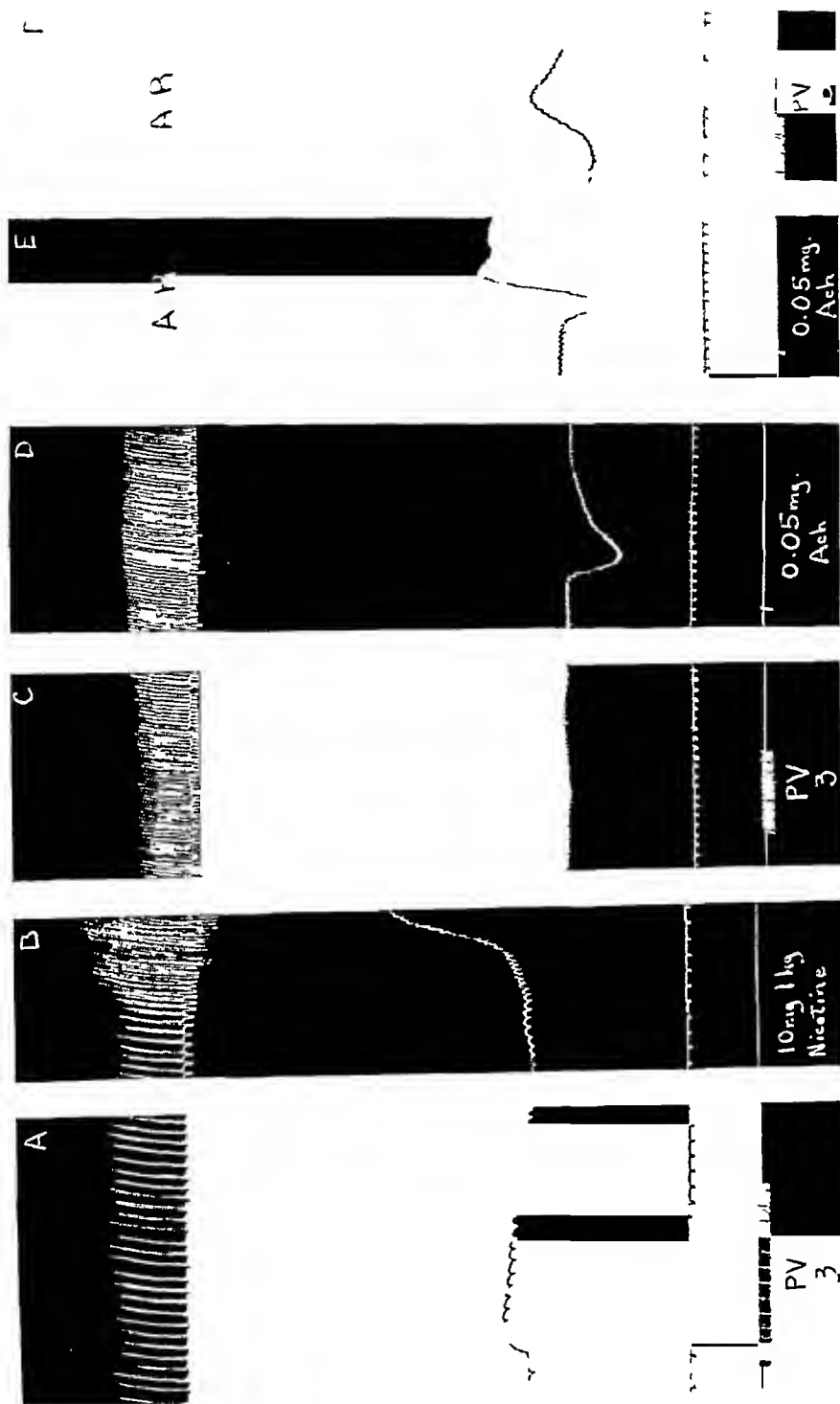


FIG 2 Dog, female, wt 10.7 kg. Nembutal anesthesia. Premedication with 10 mg per kg of atropine sulfate and 0.5 mg per kg of disopropyl fluorophosphate by vein. Upper line—respiration, second line—mean arterial blood pressure, third line—base line and time signal (time 5 seconds), lowermost line—injection marks.

A. PV 3—faradic stimulation of the peripheral end of the vagus nerve. B. 10 mg per kg of nicotine sulfate by muscle. C. 20 minutes later. PV 3 as under A. D. 0.05 mg per kg of acetylcholine chloride by vein. Note reversal of the usual acetylcholine pressor effect. E. Between D and F, 5.0 mg per kg of neostigmine by vein had been given in divided doses. Artificial respiration (A R) instituted. 0.05 mg per kg of acetylcholine. Note partial restoration of the usual ACh pressor effect. F. PV 3 as under A. Note the restoration of the abolished vagus pressor effect.

Cardiac acceleration in atropinized animals also may be produced by vagus stimulation. This may be due either to a true nicotinic effect of liberated acetylcholine, or to stimulation of accelerator fibers running in the vagus trunks, which are alleged to possess a low threshold of irritability. Cardiac acceleration produced by vagus stimulation, or by injection of nicotinic doses of acetylcholine, may be enhanced by cholinesterase inhibitors, by cocaine, and abolished by paralytic doses of nicotine and curare. (Fig 2 shows a typical experiment in which the pressor effect following peripheral vagus stimulation was abolished by a large dose of nicotine. Five mg /kg of neostigmine restored the vagus effect as well as the nicotinic effect of acetylcholine, which was likewise abolished by the large dose of nicotine.)

The cause of cardiac acceleration following the injection of large doses of acetylcholine in atropinized animals appears to be a nicotinic effect, although the experimental evidence is conflicting. McDowall (28) states that the stimulating action may be observed also following the administration of nicotine in doses sufficient to paralyze the autonomic ganglia. He believes that this is a direct action on the cardiac muscle. Hoffmann, et al (15), and McNamara, Krop and McKay (29), on the other hand, report that the positive ino- and chronotropic effects of acetylcholine are abolished by ganglionic paralyzants such as nicotine, curare, and dimethylpiperidine. The latter drugs do not modify the effect of epinephrine on the heart. The bulk of evidence, therefore, indicates that the stimulating action of acetylcholine is not a direct one on the myocardium.

Electrocardiographic analysis of the action of intravenously injected acetylcholine on the heart was first reported by Goldenberg and Rothberger (13). They observed a decrease in amplitude (notching) and even a diphasic appearance of the P waves. With progressively larger doses, A-V block (two auricular beats to one ventricular beat), with auricular fibrillation (up to rates of 1800 beats per minute) occurred. Return to normal sinus rhythm proceeded through the stages of coarser and slower fibrillation and flutter. These experiments were essentially confirmed by Noth, Essex, and Barnes (36).

In this laboratory, electrocardiographic studies were made of the effects of 50 mg /kg of intravenously injected acetylcholine in dogs narcotized with pentobarbital sodium. Some animals were premedi-

cated with 0.05 mg/kg of physostigmine. At first the control leads were taken. During and following injection a continuous tracing of Lead 2 was made. In the non-physostigminized animals there was an immediate period of complete auricular and ventricular asystole lasting about three seconds. Then followed a high-grade partial heart block, gradually subsiding, as shown by the increased number of responses of the ventricles to the auricles. Then a period of very rapid sinus tachycardia was recorded, which gradually became slower. Shortly after the onset of tachycardia, a downward RST displacement became evident. At the greatest degree of this displacement, the T wave



FIG 3 Dog, 10.8 kg. Pentobarbital sodium anesthesia. Leads 1, 2, and 3 before the injection of acetylcholine.

became inverted. With the decrease in heart rate, the RST displacement lessened and the terminal portion of the T wave became upright. The tracings contain evidence of complete suppression of both the S-A and A-V nodes, with indications that the sinus node recovers before the A-V node (Figs 3 and 4).

The appearance of the downward displacement of the RST segment represents an injury effect in the muscle closest to the endocardial surface. This injury is probably due to ischemia of the heart muscle resulting from the asystolic period and not from coronary constriction caused by acetylcholine. Katz, et al (16) have shown that this drug in the cat may cause coronary vasoconstriction, but in the dog produces only coronary vasodilation.

In dogs premedicated with physostigmine, the electrocardiographic changes are more intense. Complete asystole may last for 50 seconds. Idioventricular rhythm then appears, at first very slow (8 beats per minute), but becoming progressively faster. With the advent of idioventricular rhythm, the auricles begin to fibrillate. Later, the fibril-

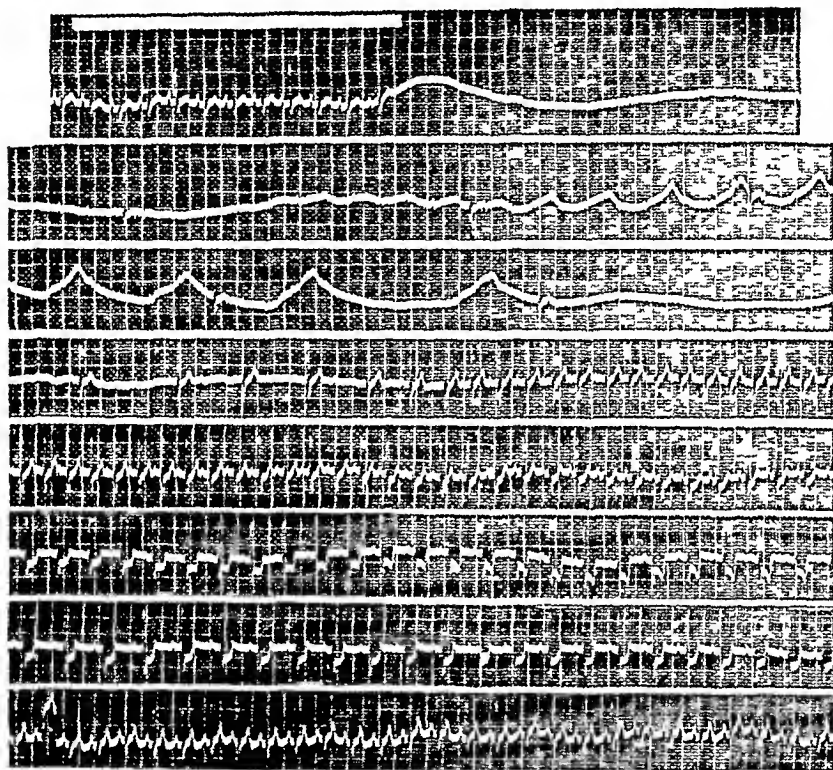


FIG. 4. Dog, 10.8 kg. Pentobarbital sodium anesthesia. Continuous tracing of Lead 2 during and after the intravenous injection of 5.0 mg/kg of acetylcholine chloride. The first seven strips are continuous, the eighth strip was taken five minutes after the seventh.

lation becomes coarser and the sinus node eventually resumes control of the auricles in the presence of complete A-V dissociation. In some instances, the A-V node never recovers, as manifested by the lack of any ventricular responses to the auricles, either when fibrillation is present, or later when the sinus node begins to initiate auricular beats. The idioventricular pacemaker is below the bifurcation of the bundle of His (Figs. 5 and 6).

Acetylcholine is the most potent vasodilator and vasodepressant agent when administered intravenously. With larger doses, the vaso-depression represents a combination of vasodilation and cardiac inhibition. Figs 7 and 8 illustrate the type and duration of the vasodepressor effects of acetylcholine, with gradually increasing doses the

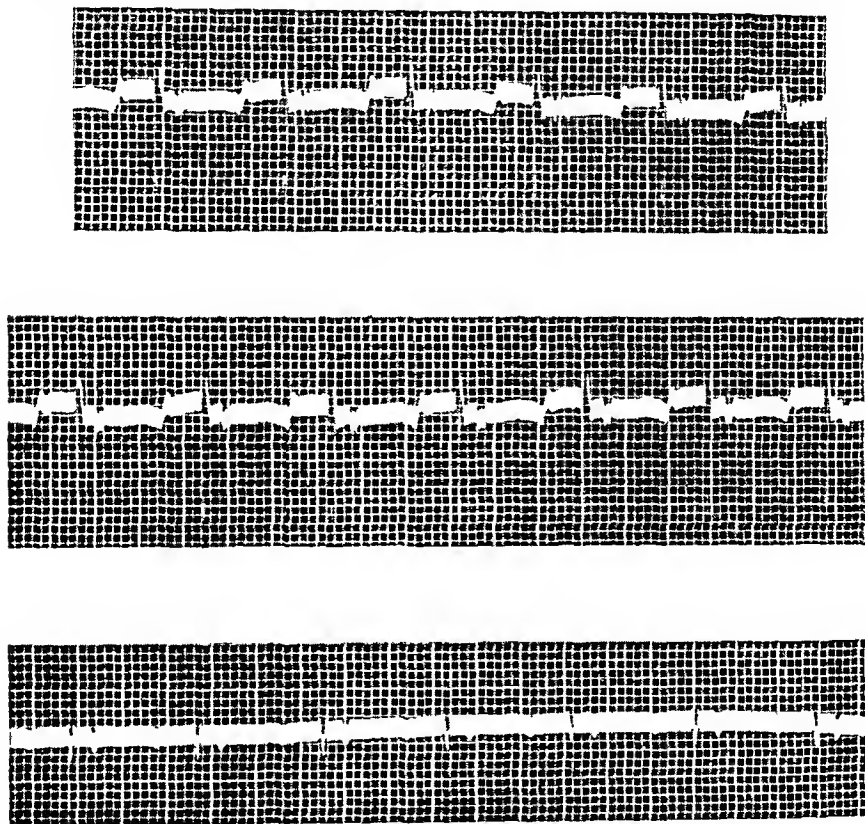


FIG 5 Dog, 11.2 kg Pentobarbital sodium anesthesia 0.05 mg/kg physostigmine sulfate by vein. Leads 1, 2 and 3 prior to the injection of acetylcholine

blood pressure fall becomes greater and the recovery period more prolonged. Cholinesterase inhibitors intensify these effects.

Acetylcholine has a nicotinic effect on the peripheral circulation, as it has on the heart. This nicotinic effect, consisting of vasoconstriction and elevation of blood pressure, is best seen if the muscarinic effects on the heart and blood pressure are prevented by previously adminis-

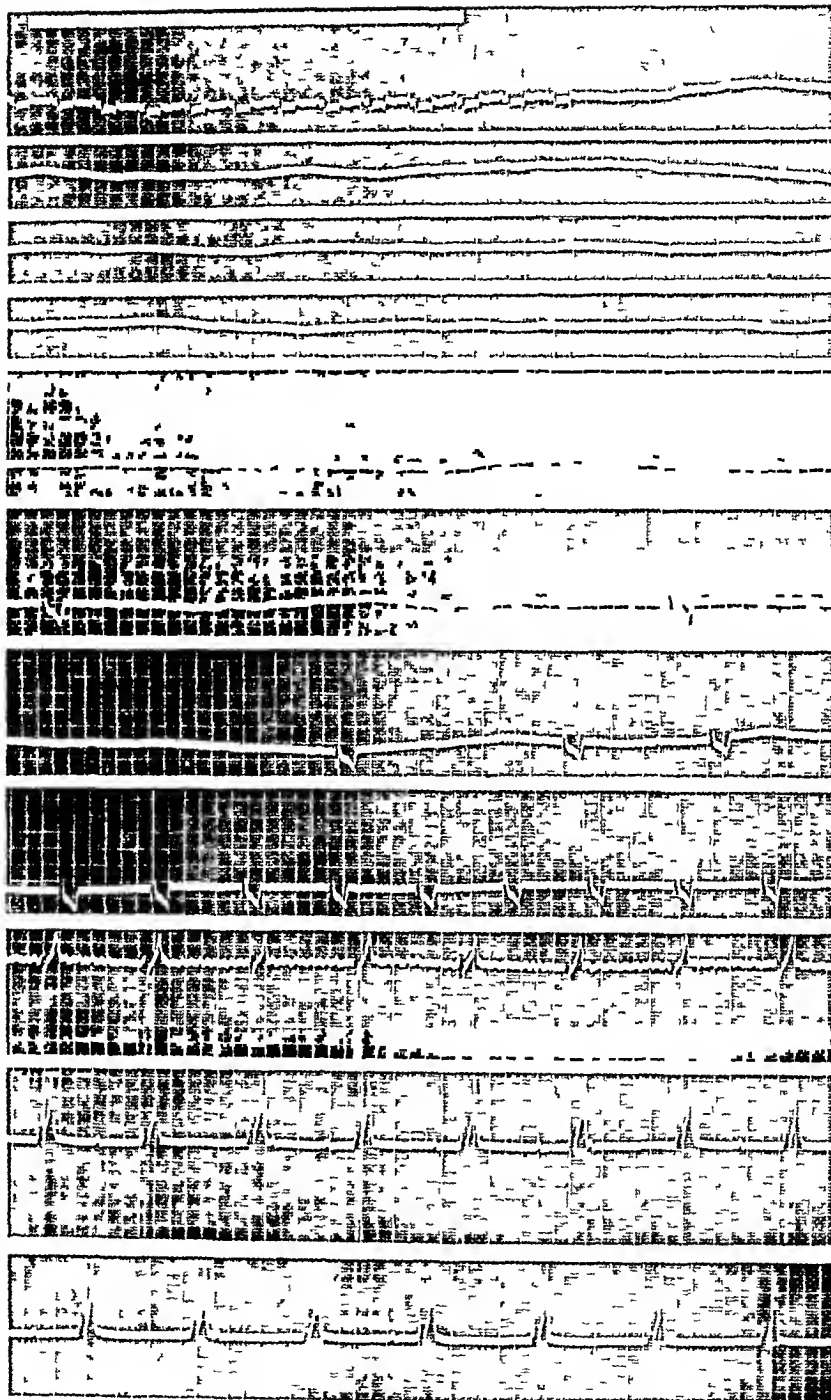


FIG 6 Dog, 11.2 kg Pentobarbital sodium anesthesia, and premedication with 0.05 mg/kg physostigmine sulfate by vein. Continuous tracing of Lead 2 during and after intravenous injection of 50 mg/kg of acetylcholine chloride. The first ten strips are continuous, the eleventh taken five minutes after the tenth.

tered atropine. It is possible, however, to obtain a secondary rise in blood pressure following the initial fall by large doses of acetylcholine in cocaineized dogs not previously atropinized (Fig. 9).

The vasopressor effect of acetylcholine is intensified by cholinesterase inhibitors and abolished by ganglionic depressants such as nicotine,

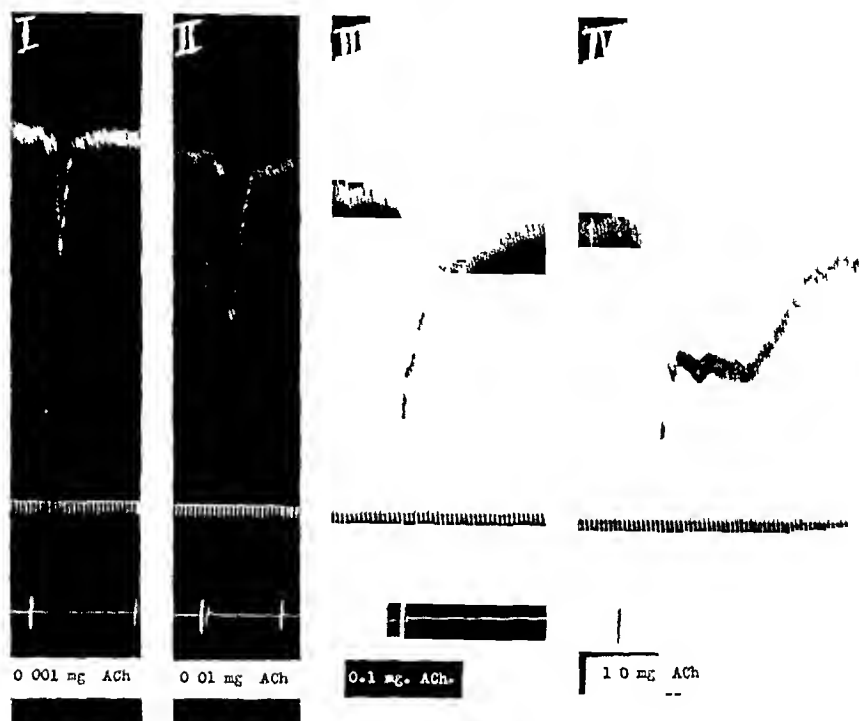


FIG. 7 Dog, male, wt 14.1 kg. 35 mg per kg of sodium pentobarbital anesthesia by vein. Upper line—mean arterial blood pressure, second line—base line and time signal (time 5 seconds), lowermost line—injection marks.

I 0.001 mg per kg of acetylcholine chloride by vein. II 0.01 mg per kg of acetylcholine chloride by vein. III 0.1 mg per kg of acetylcholine chloride by vein (Respiratory stimulation). IV 1.0 mg per kg of acetylcholine chloride by vein (Respiratory stimulation).

curare preparations, and dimethylpiperidines (Koppányi, Lnegar and Herwick, 23), (Koppányi and Vivino, 25), and (McNamara, Krop and McKay, 29). The pressor effects produced by acetylcholine are preserved following removal of the carotid sinuses, the central nervous system, the adrenal glands, the liver, and/or clamping of the abdominal



FIG. 8 Dog, male, wt 14.1 kg Continuation of Fig. 7

I 5.0 mg per kg of acetylcholine chloride by vein (Bronchial spasm, salivation, defecation) II 10.0 mg per kg of acetylcholine chloride by vein (Side effects as under I) III 15.0 mg per kg of acetylcholine chloride by vein (Side effects as under I) IV 25.0 mg per kg of acetylcholine chloride by vein (Side effects as under I) V 10.0 mg per kg of acetylcholine chloride by vein (Side effects as under I, plus micturition) VI 15.0 mg per kg of acetylcholine chloride by vein (Side effects as under I, plus micturition)

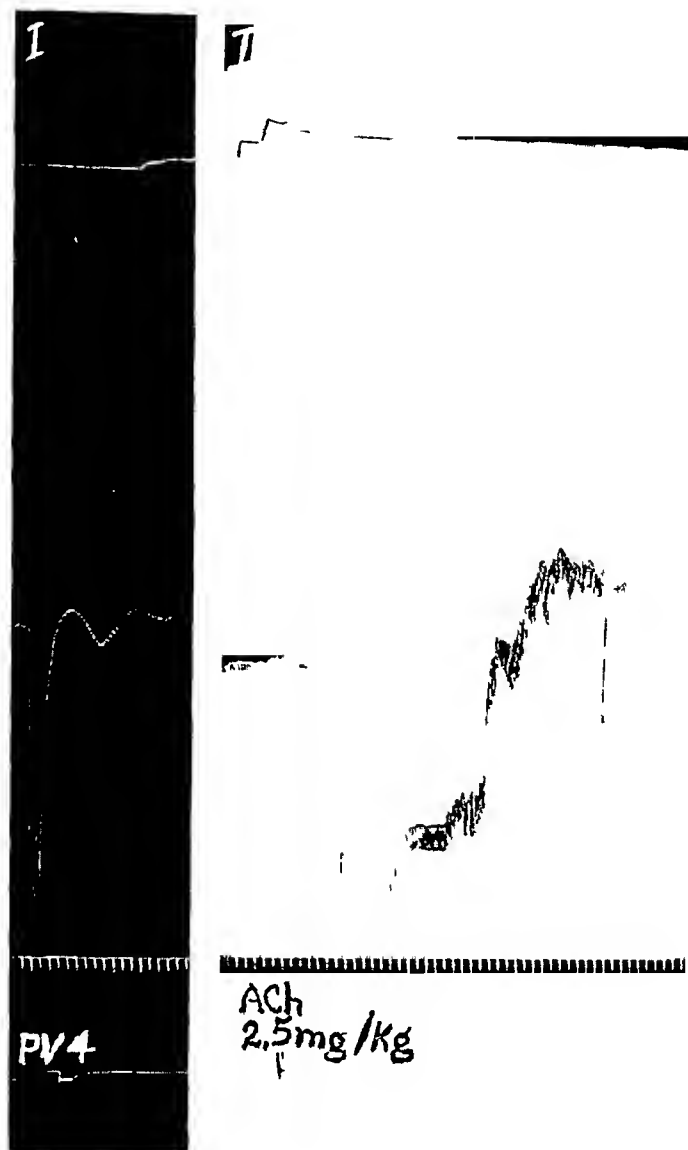


FIG 9 Dog, male, wt 17.2 kg 35 mg per kg of sodium pentobarbital anesthesia by vein 5 mg per kg of cocaine hydrochloride by muscle Upper line—duodenal motility, second line—mean arterial blood pressure, third line—base line and time signal (time 5 seconds), lowermost line—injection marks

I Faradic stimulation of peripheral vagus 4 II 2.5 mg per kg of acetylcholine chloride, by vein

aorta at the level of the diaphragm Upon the removal of the thoracic sympathetic chain and the superior cervical ganglia, acetylcholine does not produce pressor effects or other nicotinic actions following clamping of the abdominal aorta

The pressor substance, or substances, released as a result of acetylcholine injection may be transferred from one animal to another by a method of interrupted blood transfusion, producing pressor effects in a non-atropinized, but cocaineized recipient (Koppanyi, Linegar and Herwick, 23)

There is little doubt, therefore, that the same factor causing cardiac acceleration is also responsible for the pressor effect of acetylcholine McNamara, Krop and McKay, (29) made the interesting observation that calcium restores the pressor effect of acetylcholine following its abolition by nicotine or dimethylpiperidine In this connection, it may be recalled that Linegar (27) has shown that barium restores the pressor effect of acetylcholine after it has been abolished by the administration of ergotamine Large doses of antiesterases have similar effects

C RESPIRATION

Acetylcholine, even in small doses, may reflexly increase the rate and depth of respiration, due to its vasodepressor action In atropinized animals, the effects on the respiration are purely nicotinic and consist of marked but fairly fleeting respiratory stimulation, followed by depression Koppanyi, Linegar and Herwick (23) have shown that the respiratory effects of acetylcholine are greatly enhanced by previous administration of antiesterases and by cocaine Koppanyi and Linegar (22) reported that, when the carotid bodies are removed and the vagi sectioned above the ganglia nodosa, acetylcholine did not produce respiratory stimulation (in cats and dogs) Paralytic doses of ganglionic poisons (nicotine, dimethylpiperidines) diminished or abolished the "nicotinic" respiratory stimulating action of acetylcholine It is obvious, therefore, that respiratory effects of acetylcholine are due to the stimulation of the chemoreceptors which morphologically may be equivalents of sympathetic ganglia or chromaffin cells It does not necessarily follow, however, that acetylcholine is the chemical mediator to the chemoreceptors

It is remarkable that acetylcholine, even when injected intravenously (in dogs) in massive doses up to 25 mg /kg , has only a very fleeting bronchoconstrictor effect. It seldom lasts for over 30 seconds. Perhaps the nicotinic effects tending to produce bronchial dilatation oppose the muscarinic spasm. It may be interesting to note that some of the reversible and irreversible cholinesterase inhibitors, when given in large doses, may produce death by bronchial constriction even in atropinized animals, so that artificial respiration is necessary to keep them alive (Koppányi, et al , 20) and (King, et al , 17)

Weiss and Ellis (43) and also Carmichael and Fraser (8) reported that the intravenous injection of large amounts of acetylcholine (as much as 0.5 mg /kg) in human beings produced sensations of obstructed breathing, constriction in the chest, burning in the throat, and a desire to cough. Coughing was most noticeable in individuals suffering from respiratory infections. The cough could be voluntarily suppressed. When small amounts of physostigmine were given prior to acetylcholine, the symptoms were accentuated and lasted longer. In most subjects the rate and depth of respiration increased.

D SKELETAL MUSCLE

The classical researches of Brown, Dale and Feldberg (6) established that when acetylcholine is injected directly into the empty arteries of the mammalian muscle it causes contraction at not less than half the speed of the maximal motor nerve twitch. An amount of 2 gammas is adequate to produce contractions. With small doses of physostigmine (from 0.1 to 0.3 mg /kg), the response to a maximal nerve volley changed from a simple twitch to a repetitive tetanus-like effect. The tension was twice or more that of the normal twitch. Similarly, small or moderate doses of physostigmine potentiated the effect of acetylcholine on the muscle. These authors suggest that acetylcholine is involved in the chemical transmission of impulses from nerve to voluntary muscle and emphasize the importance of the choice of anesthetic for the success of such experiments.

Maurer (30), who carried out brief tetanic stimulation of the tibial nerve in narcotized cats, has shown, in accordance with other authors, that large doses of physostigmine prevent rather than enhance the muscle effects of acetylcholine. In animals anesthetized with pento-

barbital, atropinized or non-atropinized, all but the initial response to indirect stimulation of the nerve or to acetylcholine was abolished after 12 mg of physostigmine per kg. In animals anesthetized with ethyl

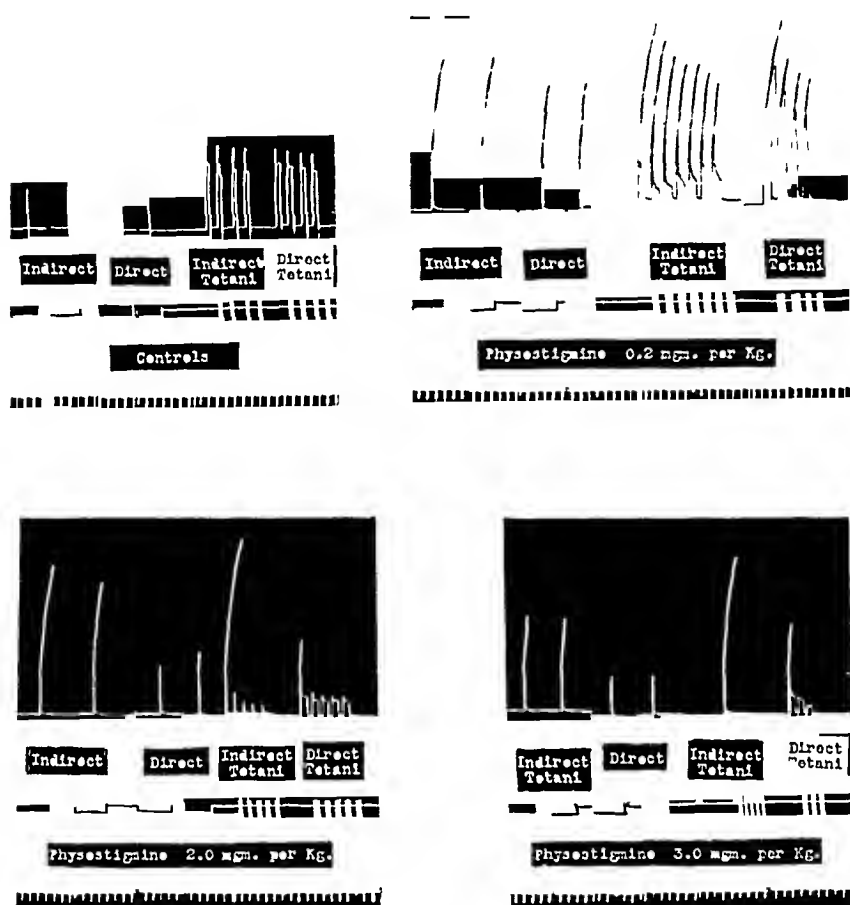


FIG 10 Cat, 2.5 kg. Anesthesia—95 per cent ethyl alcohol (7.5 cc per kg by mouth) 1.0 mg per kg of atropine sulfate by vein. Direct and indirect single shock and tetanic stimulations before physostigmine administration and following the intravenous injection of 0.2 mg per kg, 2.0 mg per kg, and 3.0 mg per kg of physostigmine.

alcohol, with or without atropine, response to indirect stimulation was greatly reduced or abolished with as little as 2.0 mg/kg of physostigmine (Fig 10). Initial responses were abolished only after from 80–100 mg of physostigmine. Continuous perfusion of the muscle

with acetylcholine solutions also reduced or inhibited the responses to tibial stimulation

Lands (26) demonstrated that the well-known phenomenon of acetylcholine-contraction of the rectus muscle of the frog is potentiated by physostigmine, abolished by curare, and to a lesser extent also by atropine. The latter drugs do not influence the response of the muscle to potassium.

Mique (32) has shown that diisopropyl fluorophosphate ("DFP") enhances the response of the frog rectus muscle to acetylcholine, but not to neostigmine, physostigmine or trimethylamine. Riker and Wescoe (38) found that many cholinesterase inhibitors potentiate the effect of acetylcholine on the mammalian skeletal muscle, but that neostigmine also possesses an independent stimulating effect.

When acetylcholine is given in large doses to anesthetized animals, fasciculations and muscle twitches are frequently observed. These effects are exaggerated after premedication with esterase inhibitors. In the unanesthetized mammal and bird, the intravenous injection of large doses of acetylcholine, from 1.5 mg/kg upward, produces extensor rigidity of the limbs, but whether these effects are muscular, central or asphyxial still remains to be determined. (According to Duke-Elder, extra-ocular striated muscles contract following injection of acetylcholine by vein.)

E EYE

The muscarinic action of acetylcholine on the eye should result in constriction of the pupil, spasm of accommodation, false myopia, and lowering of intraocular tension. As a matter of fact, acetylcholine has been given clinically as a miotic by subconjunctival injection. According to Molitor (33), acetylcholine when instilled into the rabbit's conjunctival sac, even in a 5 percent solution, produces no miosis except when the eyelid is gently massaged following instillation. (ACh, due to its vasodilator effect, is said to have distinct therapeutic effects in tobacco amblyopia and retrobulbar optic neuritis (Duggan).)

In a number of unanesthetized rabbits, we endeavored to elicit the muscarinic effects of acetylcholine on the eye upon instillation of a 5 percent solution into the conjunctival sac, but were unable to observe miosis even after prolonged massage. When acetylcholine was injected

intravenously into unanesthetized dogs, in no instance was it possible to demonstrate pupillary constriction. Following injection of doses of from 0.5 to 15.0 mg/kg mydriasis occurred in every case. The only way in which it was possible to produce miosis in rabbits was by Koppány's method (19) of intraocular injection of 0.05 cc of a 5 per cent acetylcholine solution into the anterior chamber. This resulted in maximum miosis within three minutes after injection. The intraocular administration of solutions of similar pH ranges produced no comparable effects.

The nicotinic effect of acetylcholine on the eye consists of pupillary dilation, slight exophthalmos, widening of the palpebral fissure, and withdrawal of the nictitating membrane. These effects can be demonstrated in atropinized animals with doses of acetylcholine of about 0.5 mg/kg or more. If atropinized animals are given anticholinesterases, smaller doses of acetylcholine likewise produce nicotinic effects. Whether the pupillary dilatation seen following intravenous injection of from 1.0 to 25.0 mg/kg of acetylcholine into non-atropinized animals is due to anoxia (cardiac asystole and bronchospasm), to excitement, or to true nicotinic effects, has not been determined.

The action of acetylcholine on the eye is thus mainly nicotinic. This point may be illustrated in pigeons receiving 5.0 mg/kg of acetylcholine by vein, in which, unlike in dogs and rabbits, a prompt pupillary constriction was observed in each case. Miosis in pigeons is, of course, a nicotinic and not a muscarinic effect, since the sphincter of their iris consists of striated muscle (Koppány and Sun, 23).

Sympathetic ganglia are involved in the production of mydriasis and of other signs of sympathetic ocular stimulation, since Bacq (2) and also Koppány, et al (20) have been unable to detect signs of nicotinic stimulation of the dilator muscle of the iris and of the orbital smooth muscles following complete sympathetic ganglionectomy, or following the injection of paralytic doses of nicotine (Koppány, et al 20).

It should be pointed out that proven miosis with acetylcholine has been produced only by subconjunctival, intraocular or intracarotid (homolateral miosis) injection (Battro and Lanari (3, 4)). This indicates clearly that large doses of acetylcholine cause miosis only when they come in contact with the parasympathetic effectors in the sphincter muscle before they can affect the sympathetic ganglia.

F URINE SECRETION

Intravenous injection of acetylcholine (Pickford (37)) causes a temporary inhibition of water diuresis in unanesthetized dogs. This inhibitory effect is absent after the removal of the posterior lobe of the hypophysis. Pickford postulated that this inhibition is produced by an effect of acetylcholine on the central nervous system, leading to an "outpour" of the postpituitary antidiuretic hormone into the circulation. In order to test the hypothesis that acetylcholine plays a rôle in transmitting the impulse in the hypothalamus, she injected the drug into the supraoptic nucleus of anesthetized dogs whose kidneys had been denervated and were in a state of water diuresis. This procedure resulted in a prompt inhibition of urine secretion. This action was prolonged by the addition of physostigmine, which alone also causes some inhibition of water diuresis. The inhibition of water diuresis is neither circulatory nor renal since in every case it is prevented by hypophysectomy.

The inhibition of water diuresis is not inconsistent with an effect of large doses of acetylcholine in unanesthetized dogs, resulting in micturition. This is undoubtedly due to a muscarinic effect on the body of the bladder and a subsequent rise in the intravesical pressure.

Note The intravenous injection of small or moderate doses of acetylcholine into multiparous or pregnant rabbits causes a firm spasm of the uterus. This muscarinic effect is prevented by previous atropinization. If, however, large doses of acetylcholine (0.5 mg/kg or more) are injected into these atropinized animals the same type of spasm is elicited. This is a nicotinic action due to stimulation of the motor sympathetic supply to the uterus. It is potentiated by physostigmine and abolished by large doses of nicotine. Here we have an example where the outward manifestation of muscarinic and nicotinic effects are exactly alike, while the mechanism of action is different.

G CENTRAL NERVOUS SYSTEM

Early observers studying the central action of acetylcholine generally reported depressant effects from acetylcholine. Schweitzer and Wright (39) and other authors demonstrated that under certain conditions this drug depressed spinal reflexes. Somewhat later, however,

it became increasingly obvious that acetylcholine, particularly in physostigminized animals, produced on local application to the cerebral cortex, or following intracarotid injection, tremors, extensor rigidity of the limbs, excitement, and even convulsions, indicating facilitation of transmission at cortical synapses

The cortical stimulating action of acetylcholine and of some cholinesterase inhibitors was further substantiated by electrocorticographic studies. Acetylcholine produced on local application to the cortex, in animals not previously treated with cholinesterase inhibitors, a slight reduction in amplitude of the slow waves (Miller, Stavratsky and Woon-ton, 31). In animals previously treated with physostigmine, acetylcholine, when applied in small amounts (0.2-1.0 percent solutions) to the cortex, produced large, rapid spikes, prevented or abolished by atropine. Chatfield and Dempsey (9), on the other hand, found no noticeable changes in action potentials when acetylcholine alone was applied to the cortex. If such application, however, followed treatment of the cortex with a one percent solution of neostigmine, the spontaneous bursts increased in size and the individual potentials within the burst became larger and sharper, assuming a spike shape. Spikes also appeared between the bursts until a continuous series of spikes appeared. Such spikes appeared upon local application to the "acoustic," "somesthetic" and "motor" cortex, but not to the "association" cortex.

Brenner and Merritt (5) found that only 2.5 to 10 percent solutions of acetylcholine applied to the cortex induced high potential slow and spiked formations. Darrow, et al (10) suggest that tachycardia during hyperventilation is responsible for the slowing of the brain waves. Stimulation of the cut facial nerves (cholinergic innervation to the pial vessels) reduced or prevented slow waves. Physostigmine acted similarly, while atropine enhanced the effects opposed by facial nerve stimulation or by physostigmine. These authors demonstrated a parasympathetic influence on the electrical activity of the cortex and implied that such influence may affect cerebral metabolism and circulation.

Brenner and Merritt (5) and Darrow, et al (10) suggest that small amounts of acetylcholine have an indirect muscarinic (vascular, etc.) action on the cortex, while larger amounts have a direct nicotinic action

on synaptic transmission In any event, it appears that acetylcholine applied locally to the motor cortex or administered by injection produces EEG changes characteristic of "grand mal" epilepsy and of the effects of convulsant drugs

Stavraky (40) and Fischer and Stavraky (12) have shown that removal of various portions of the cerebral hemispheres resulted in a prolonged sensitization of the remaining parts of the central nervous system to intravenous doses of acetylcholine Motor responses following such injections were especially marked Unpublished results demonstrate that dogs anesthetized with 25 mg /kg of sodium pentobarbital, following the intravenous injection of 5 mg /kg of acetylcholine, showed phonation and motor activity Occasionally they lifted their heads and attempted to get up (Possible denarcotizing effect)

Bulbring and Burn (7) have shown in a series of well controlled experiments that acetylcholine, contrary to earlier observations, is a spinal stimulant When this agent was injected into the spinal cord, discharge of motor impulses occurred with doses as small as 1 gamma They found that atropine prevented or terminated the effect of acetylcholine, physostigmine, and neostigmine on the spinal cord Wescoe, Green, McNamara and Krop (44) recently showed that the electroencephalographic and central excitatory, including convulsive, effects of DFP are abolished by atropine They assume that DFP acts solely by cholinesterase inhibition and thus conclude that atropine antagonizes the central effects of acetylcholine

Reasoning by analogy, it is possible that hemorrhages in the grey substance of the central nervous system, accompanied by diffuse gliosis and subsequently by development of glial nodules, glial scars, and neurone depletion, are also produced by acetylcholine, as they are produced by carbamylcholine and by the combined administration of choline and physostigmine (Davis and Fletcher, 11) Since the subcutaneous administration of carbamylcholine (0.01 mg /kg hypodermically daily for a month) also produced hyperchromic anemia, it is not impossible that the neuropathological changes produced in dogs are related to this condition

H ABSORPTION AND TOXICITY

It is not surprising that the data concerning the absorption and the oral, subcutaneous and intramuscular toxicity of acetylcholine are contradictory and confusing. The intravenous toxicity, because of the existence of transport cholinesterase, depends upon the rate of injection. Therefore, unless the rate is strictly controlled, vastly different results will be obtained. Hence, the disagreement between Weiss and Ellis (43) and Carmichael and Fraser (8) on the effects of intravenously injected acetylcholine on the human heart.

Molitor (33) published data on the oral, subcutaneous and intravenous LD_{50} in rats and mice. His findings should be supplemented by experiments on other species, including higher mammals, as well as birds, reptiles, amphibia and fishes. Preliminary experiments carried out in this laboratory indicate great species-specific differences in the resistance to the toxic effects of acetylcholine.

The rates of absorption from the alimentary tract, subcutaneous tissues and skeletal muscle, as well as the corresponding toxicity, depend upon the transport cholinesterase. Probably small doses of DFP are sufficient to inactivate this esterase, and the absorption and toxicity studies (employing well defined end-points) should also be repeated in DFP-treated animals. It seems particularly desirable to obtain data on the absorption and toxicity of acetylcholine administered by the intratracheal route ("acetylcholine aerosol" administered through a nebulizer).

I ACETYLCHOLINE AND THE CHOLINESTERASE INHIBITORS

A pharmacological study of acetylcholine cannot be divorced from the cholinesterase problem since it is established that the fate of injected acetylcholine is hydrolysis by the cholinesterases. Many drugs that are known to inhibit cholinesterase activity in vitro prolong the action of injected acetylcholine in the body. In fact, all known pharmacological actions of acetylcholine, both of the muscarinic and nicotinic types, are potentiated by cholinesterase inhibitors, such as physostigmine, neostigmine, DFP, HETP, TEPP, or hexaocetyltetraphosphate (HOTP). The potentiation is due to the effect on the essential cholinesterase, which includes both the transport cholinesterase and the specific cholinesterase most prominently involved.

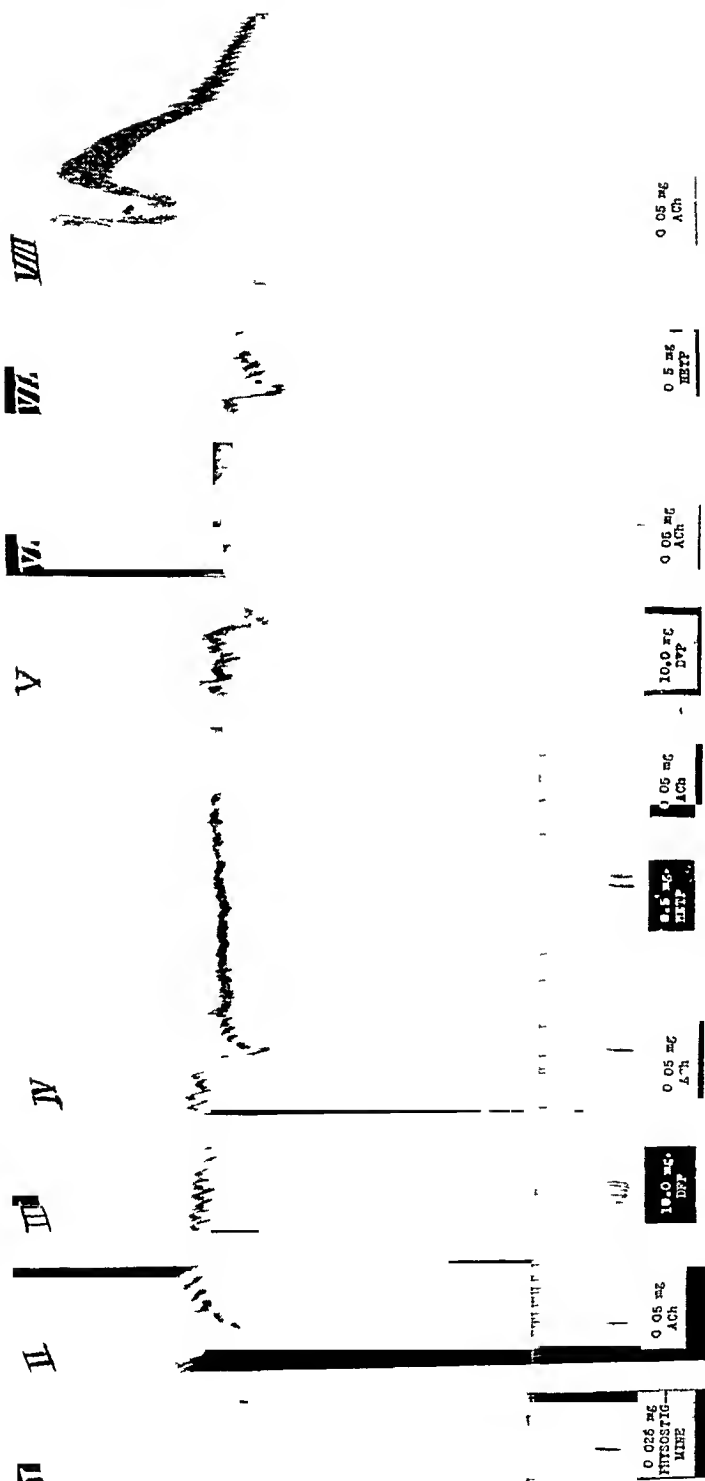


FIG 11 Dog, male, wt 13.3 kg 35 mg per kg of sodium pentobarbital anesthesia, by vein, premedication with 10 mg per kg of atropine sulfate by vein

I 0.025 mg per kg of physostigmine salicylate by vein II 0.05 mg per kg of acetylcholine chloride by vein III Ten minutes later, 10 mg per kg of DFP by vein IV Twenty minutes later, acetylcholine as under 2, followed by 0.5 mg per kg of HCTP Five minutes later, acetylcholine as under 2 V Three hours later, 10 mg per kg of DFP, by vein VI Twenty minutes later, acetylcholine as under 2 VII Ten minutes later, 0.5 mg per kg of HCTP by vein VIII Five minutes later, acetylcholine as under 2

Relatively small doses of DFP are sufficient to inactivate the transport cholinesterase, and additional doses of DFP or of other anticholinesterases further enhance the acetylcholine effect by their actions on the specific cholinesterase. The effects of physostigmine and neostigmine on acetylcholine potentiation are probably only partly due to

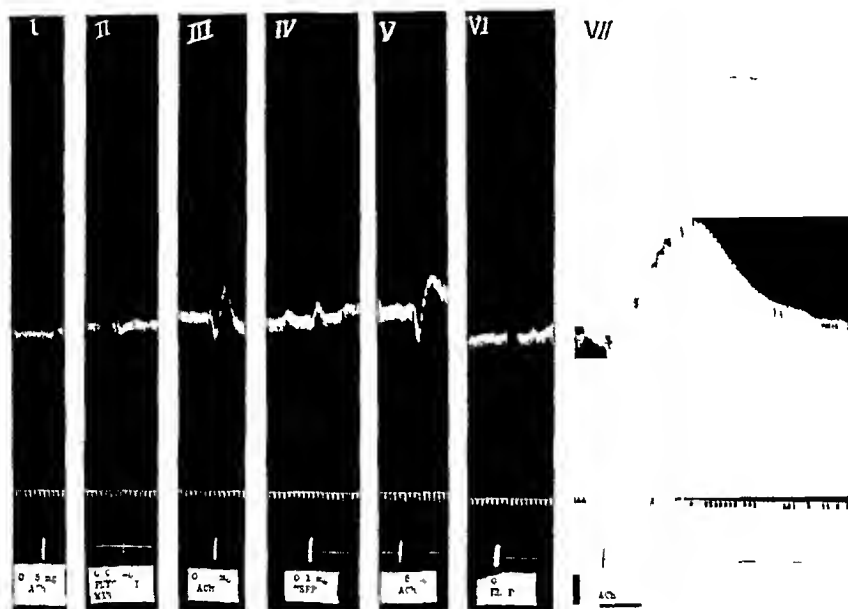


FIG 12 Dog, male, wt 12.7 kg. 35 mg per kg sodium pentobarbital anesthesia. Premedication with 10 mg per kg of atropine sulfate by vein. Upper line (on 7) duodenal motility, second line—mean arterial blood pressure, third line—base line and time signal (time 5 seconds), lowermost line— injection marks.

I 0.05 mg per kg acetylcholine chloride by vein. II 0.05 mg per kg of physostigmine salicylate by vein. III Ten minutes later, acetylcholine as under I. IV 0.1 mg per kg of TEPP by vein. V Thirteen minutes later, acetylcholine as under I. VI One hour later, 0.5 mg per kg of HCLTP by vein. VII Acetylcholine as under I.

antiesterase activity, because minimal and subminimal doses of these drugs enhance beyond expectation the acetylcholine-potentiating actions of DFP (Koppanyi, Karczmar and King, 21).

The measurements of the pressor (nicotinic) responses to standard doses of acetylcholine, in the presence of varying concentrations of different anticholinesterases, can be used as an *in vivo* estimate of the

inactivation of the total (essential) cholinesterase. The highest pressor response under optimum conditions is taken as approximately 100 percent inhibition of the total cholinesterase. It can also be shown by this method that the very high threshold of the ganglia to intravenously injected acetylcholine is due to cholinesterase protection, because in atropinized animals with optimum doses of TEPP as small a dose of acetylcholine as 0.8 gamma/kg is sufficient to elicit pressor responses (Koppanyi, Karczmar and King, 20).

Koelle (18) has shown that premedication with physostigmine diminishes the toxicity of DFP. His results were not only confirmed but were extended in this laboratory. Very small doses of physostigmine or of neostigmine (0.05 mg/kg or less), scarcely sufficient to permit the production of pressor effects following intravenous injection of acetylcholine in atropinized animals, prevent the development of pressor effects of usual magnitude that may follow the administration of large amounts of DFP, HEPT, or TEPP (Figs 11 and 12). On the other hand, HETP and TEPP do not fully prevent DFP effects (21). As stated above, the administration of DFP never interferes with further potentiation of acetylcholine by other cholinesterase inhibitors.

This paper does not deal with the physiological rôle of acetylcholine, and therefore does not attempt to evaluate the relative importance of cholinesterase versus the acetylcholine precursor of Abdon (1). There are no pharmacological methods available to modify the breaking-down of the precursor into free acetylcholine, or for the stimulation of the resynthesis of the precursor which, according to Abdon (1), requires energy, probably delivered from the dephosphorylation of phosphocreatine and adenosinetriphosphoric acid.

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DISCUSSION

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The questions I would raise regarding the physiological functioning of acetylcholine are not intended as criticism of Dr Koppanyi's excellent exposition, but rather as an attempt to find basis for a neurophysiological interpretation

In treating of the effects of acetylcholine, Dr Koppanyi correctly emphasizes the frequency with which autonomically paradoxical, or so-called "nicotinic" effects, are elicited These effects are the opposite of the direct of "muscarinic" changes commonly associated with parasympathetic function He describes the nicotinic effects specifically in the case of the alimentary tract, the heart, the vasomotor system, respiration, the eye, and the central nervous system Similar effects have been noted by us in studies of autonomic influences on the central nervous system For example, we have repeatedly noted in human subjects that *slowing* of heart rate following atropine was likely to be attended by increased regularity of alpha and decrease of slow waves in the electroencephalogram (5) A similar effect on the EEG has been noted by Obrador (11)

Dr Koppanyi repeatedly emphasizes the importance of the nicotinic action of acetylcholine, primarily, apparently, as an effect on ganglionic transmission On the other hand, I would take this opportunity to emphasize the rôle of the moderator nerves In many instances, at

least, it appears that central and autonomically paradoxical effects are understandable as a homeostatic action, overaction, or change in sensitivity of so-called "servile" or "feed-back" mechanisms which contribute to homeostatic regulations by the moderator nerves. The steps taken to eliminate these influences experimentally have in some instances been specified by Dr. Koppanyi,—and in some cases, after the elimination of possible moderator influences, the effect remained. The implication is that in such cases moderator influences are unimportant. I wonder if that follows.

Moderator mechanisms are many and serve common homeostatic functions, often they are apparently susceptible in the same way to the same pharmacologic influences. Is it surprising, then, that the elimination of one or several of these mechanisms does not eliminate the function? Under these conditions failure to eliminate a function by surgery is no proof that the structure did not contribute something to the total effect.

There are many moderator mechanisms concerned with autonomic and cerebral regulations, and of these carotid sinus is only one notable example. In addition to the carotid sinuses there are, of course, the carotid bodies, the aortic arch receptors, the abdominal vasotatic receptors, intraventricular or intracisternal mechanisms, and apparently also sensitive regions in the hypophysis and hypothalamus. In the same general category, likewise, must be added the homeostatic effects of autonomic agents upon ganglionic transmission, as neurophysiologically demonstrated by Marrazzi (10). Removal of the carotid sinuses and section of the vagi would eliminate some but not all of these. Bain, Irving and McSwiney (1) have demonstrated the possibility of abdominal inhibitory effects (of stimulation) upon the parasympathetic which do not involve the vagus. And some moderator effects are produced via the sympathetic.

In the case of respiration, Dr. Koppanyi notes from his own studies with Linegar (9) that the "nicotinic" effects are abolished after the carotid bodies are removed and the vagi sectioned above the ganglion nodosum. One type of moderator mechanism is here implicated and others presumably eliminated by surgery. Section of the vagi presumably here disposes of the aortic receptors and possibly eliminates effects from the "vasotatic" receptors identified by Heymans, Bouck-

aert, Faber, and Hsu (8b) and others. The question not answered is whether these mechanisms produced nicotinic effects on respiration before surgery. It is known that acetylcholine and related substances may decrease the regulatory response of the carotid sinus (Heymans, Bouckaert, Farber and Hsu (8a)), thereby decreasing inhibition of sympathetic activity and permitting increased blood pressure or other sympathetic effects.

In the case of vasomotor changes, Dr. Koppanyi notes that "nicotinic" effects of acetylcholine on blood pressure persisted after removal of the carotid sinuses, the central nervous system, the adrenals, the liver, and/or clamping of the abdominal aorta, and that the effects were eliminated only by removal of the sympathetic chain and superior cervical ganglia, or by drugs preventing sympathetic transmission. Here the Marrazzi effect and a true nicotinic action is possibly involved. But the question still remains, is it possible that a Heymans desensitization (8a) of the moderator nerves by acetylcholine contributes under normal conditions?

Again, in the case of the pupil, Dr. Koppanyi's results point to effects of acetylcholine by way of nicotinic action on the ganglia. This again, however, does not indicate that nicotinic action is the sole possible source of paradoxical effects. Gellhorn, Darrow and Yesenick (7) demonstrated in cats marked dilatation of the pupils during fall in blood pressure following amyl nitrite. The effect persisted after carotid sinus denervation and section of the vagi. The inverse relation to, and the apparent dependence upon blood pressure changes, is not readily explained as a nicotinic ganglionic effect. It rather suggests some moderator nerve response to pressure not dependent upon the vagus. An abdominal effect via pathways such as demonstrated by Bain, Irving and McSwiney (1) to the third nerve nucleus might satisfy the requirement.

Effects upon the central nervous system likewise involve possible activity of feed-back mechanisms. In this respect I must not miss the opportunity to correct a possible misinterpretation: no one I know has implied that tachycardia *causes* slow waves in the electroencephalogram. In our laboratory, we have suggested the possibility that the cardiac acceleration which occurs in compensation for a fall in blood pressure during hyperventilation may indicate an inhibition of the

the central action of DFP has yet been studied in relation to the possible influence of hyperventilation. In view of the action of physostigmine, it seems not unlikely that doses of DFP too small to produce appreciable effects on a normally functioning nervous system might cause marked reduction of high potential slow electroencephalographic activity following hyperventilation.

In this connection, there is a further question raised by the pre-publication observation of Koppanyi, Karczmar and King that physostigmine and neostigmine "enhance beyond expectation the acetylcholine protective action of DFP." Is it possible that this implies an action of these substances directly upon the receptors as well as on the enzyme?

Furthermore, is there proof that the substance found in the blood stream or perfusates from structures treated with anticholinesterase is acetylcholine, or does it only produce some acetylcholine-like effects?

With these observations in mind as questions, but not as criticisms, I commend to you Dr. Koppanyi's excellent paper.

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IV CONCERNING THE MODE OF ACTION OF ACETYLCHOLINE¹

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The approach to the problem of the physiology of acetylcholine (Ach) has been like that to most problems in physiology. Beginning with the early work of Reid Hunt, Sir Henry Dale and Otto Loewi, the boundaries of our knowledge have been pushed back slowly. The viewpoint of any single investigator or group of associated workers has usually been a relatively narrow one, with attention centered on some specific aspect of the problem.

During the past twenty-five years much has been learned about the quantitative distribution of Ach in animal tissues, about its synthesis and its destruction, both *in vivo* and *in vitro*, and about the reaction of animals and their parts to Ach. However, we have very little information concerning the fundamental mode of action of Ach. When Ach is applied to motor end-plates, autonomic ganglia or heart muscle, we know the nature of the responses, but we know little concerning the series of events leading up to the response. Much attention has been paid to the rôles of Ach as a transmitter substance at synapses and neuromuscular junctions, and recently to a possible rôle in conduction along the axon, but little attention has been given to the physiology of acetylcholine in non-nervous tissues. Since the Ach cycle is such a prominent feature of the nervous systems of all animals, both invertebrate and vertebrate, there is a tendency to think of Ach only in connection with the functioning of cholinergic neurons. However, its occurrence in plants, in protozoa, and in such organs as spleen and placenta suggests that it may play some general rôle in cellular metabolism. If this more general rôle was an involvement in inter- and intra-cellular communication in various tissues, the nervous system, concerned as it is with rapid coordination, might well be expected to possess the Ach cycle as a prominent and characteristic feature.

¹ A portion of the work reported in this paper was supported by a grant from the U. S. Public Health Service.

A broader viewpoint and new approaches to the problem of the physiological functions of Ach are needed. These might lead to a better understanding of the manner by which Ach acts on cells. This paper will be concerned with preliminary and largely indirect studies which have been undertaken by ourselves and others with these needs in mind. Some of the suggestions which are to be offered are of a highly speculative nature. The material will be presented under the following headings

- (1) Ach and cellular respiration
- (2) Ach as a possible trophic substance
- (3) A suggested mechanism of Ach action

1 ACH AND CELLULAR RESPIRATION

Our interest in Ach and cellular respiration was first aroused through an attempt to relate Ach levels of different parts of the nervous system with other known characteristics of these parts. It was known that Ach levels differed greatly in different parts of the mammalian nervous system (MacIntosh, 1941, Welsh and Hyde, 1944a). In the central nervous system the amounts of Ach are smallest in the cerebellum and cerebral cortex and increase in the brain stem and the medulla. Motor nerves and autonomic ganglia contain more Ach than any part of the central system, while Auerbach's plexus of the small intestine was estimated by Welsh and Hyde (1944b) to have more Ach than any other nervous tissue that has been assayed about 5000 times as much Ach being found in Auerbach's plexus of the guinea pig and rabbit intestine as is found in the cerebellum. It was pointed out by Welsh and Hyde that the order of increasing resistance of the parts of the mammalian nervous system to anoxia and hypoglycemia is essentially the same as the order of parts arranged according to increasing amounts of Ach per unit weight. Those parts which are least resistant (cerebellum and cortex) are low in Ach, and those which are among the more resistant (spinal nerves and autonomic ganglia) are high in Ach, while the neurons of the myenteric plexus, shown by Cannon and Burket (1913) to be the most resistant of all vertebrate neurons to anoxia, have extraordinarily high levels of Ach. The correlation is so good that one is led to consider the possibility that stores of Ach help

to preserve the functional integrity of neurons during periods when, through oxygen or glucose lack, new Ach cannot be synthesized

This line of investigation led to speculation concerning Ach and cellular respiration. Would a deficiency in Ach production affect the rate of oxygen consumption of cells? Earlier work (Deutsch and Raper, 1936, 1938) has shown that the addition of Ach to respiring slices of sub-maxillary gland tissue increases the rate of O_2 uptake. Abdon and Borghin (1947) have shown a lowered level of oxidative metabolism of rat skeletal muscle in choline deficiency. We are now carrying on experiments with rats and certain insects on choline and pantothenic acid deficient diets, in an attempt to produce acetylcholine insufficiency. *Tenebrio* (meal worm) larvae are sensitive indicators of pantothenic acid and choline deficiency, their growth and respiration being markedly reduced on deficient diets. Preliminary experiments indicate that Ach levels are abnormally low, but it is not yet possible to say that this is a direct cause of slower growth and lowered metabolism. However, one is led to further speculation concerning a possible trophic rôle of Ach.

2 ACH AS A POSSIBLE TROPHIC SUBSTANCE

The effects of denervation on the structures deprived of their nerves is well known. In the case of structures innervated by cholinergic neurons there is at first a greatly increased sensitivity to Ach and subsequent atrophy. Cannon (1939) expressed this general phenomenon in what he termed a "Law of Denervation": "When in a series of efferent neurones a unit is destroyed, an increased irritability to chemical agents develops in the isolated structure or structures, the effect being maximal in the part directly denervated."

Likewise, studies such as that of Ward and Kennard (1942), indicating that cholinergic drugs hasten the recovery of function following lesions of the central nervous system, call attention to the possibility that Ach may also be involved in processes of regeneration.

A preliminary report (Welsh, 1946) has been made of a study of degeneration and regeneration in planaria. Ach blocking agents such as atropine induce degeneration of the head of planaria and slow the process of regeneration of pieces. Prostigmine delays the degeneration produced by atropine, while prostigmine and pilocarpine hasten the regeneration of pieces of these flatworms.

The brief references in this and the preceding section to possible rôles of Ach in processes other than conduction and transmission in the nervous system are made primarily to encourage a broader view of the physiology of Ach. Possibly Ach may help to regulate cellular metabolism and perhaps thereby act as a trophic substance. Final and convincing evidence for or against this will be obtained only through the concerted efforts of many investigators.

3. A SUGGESTED MECHANISM OF ACH ACTION

When Ach in appropriate concentrations is applied by close arterial injection to an intact organ, innervated by cholinergic nerves, or in most instances to the same organ or its part after isolation, a response characteristic of the organ or tissue may be observed, glands secrete, muscles contract, melanophores expand, neurons discharge. It is usually possible to demonstrate that Ach in low concentrations initiates a response or has an excitatory action, while at higher concentrations the response is depressed or inhibited. Since the excitatory action on one type of organ or tissue (e.g. smooth muscle of the intestine) may occur over a wide concentration range and be easily recorded or observed, while for another tissue or organ the excitatory range may be narrow and the inhibitory effects prominent (e.g. vertebrate or molluscan heart muscle), it has become customary to think of Ach as having two types of action—excitatory and inhibitory (or paralytic), separate and distinct from one another. If it could be shown that these two actions of Ach are quantitative and not qualitative, the task of solving the problem of Ach action would be simplified. No attempt has been made to gather conclusive evidence for this latter view, but from the work of McDowall (1946) and others on the vertebrate heart it would appear that Ach in small doses has an excitatory action at concentrations below those producing inhibition. In the presence of atropine or methylene blue, in amounts which only partially block Ach action, the excitatory effects are pronounced.

A similar excitatory action of Ach on the heart of the mollusc, *Venus mercenaria*, may occasionally be observed before the characteristic negative inotropic effect sets in. With choline, the excitatory action may be marked and may occur over a wide range of concentrations (Fig. 1). It is of interest that Thimann (1937) finds that growth acceleration and inhibition by auxins are but quantitative effects, and

concludes that the mode of action of auxins on roots and stems is not qualitatively different

If the possibility is granted that wherever Ach acts the mechanism of action is fundamentally the same (see Fig 2), we may then proceed to examine certain facts concerning Ach action and to devote some space to speculation, in the hope that better experiments may be designed to augment our knowledge of this aspect of its physiology

Some of the things that are known about Ach action will be illustrated by a study of its action on a highly sensitive invertebrate organ,

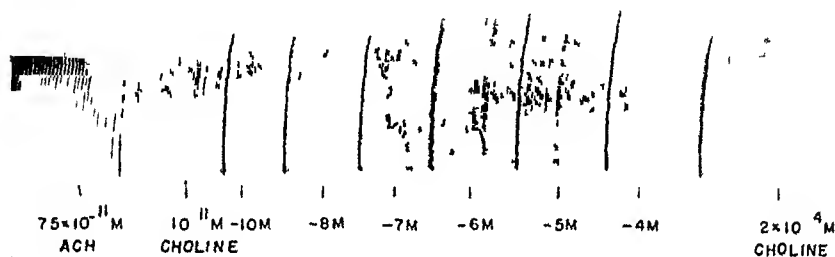


FIG 1 RESPONSE OF ISOLATED HEART OF VENUS MERCENARIA TO ACETYLCHOLINE (ACH) AT FIRST ARROW

This was an unusually sensitive preparation. At following arrows increasing amounts of choline chloride were applied, all producing an increase in amplitude until 10^{-5} choline chloride was reached which produced a just perceptible decrease in amplitude. Further increases produced more pronounced negative inotropic effects.

the ventricle of *Venus mercenaria*, the hard shell clam or quohaug. As first shown by Prosser (1940), this smooth muscle structure is innervated by nerves which are cholinergic and is a sensitive indicator for Ach. We find that the isolated ventricle may, at times, be excited by concentrations of Ach of the order of 10^{-6} μg Ach per ml of perfusion fluid. The threshold concentration for inhibition is usually between 10^{-5} and 10^{-4} μg Ach/ml, and complete stoppage of the heart occurs at concentrations about 50 times the threshold concentration for inhibition. This order of activity suggests that an enzyme system is directly involved in the action of Ach, but we shall return to this point later.

Few substances normally produced in an animal act in lower concentrations than Ach, and there appears to be no other choline ester or related substance that is more active than Ach if differences in stability to cholinesterase are taken into consideration. The Venus heart has very little cholinesterase as shown by Jullien *et al* (1938) and Smith and Glick (1939). This accounts for the very slight potentiation of Ach action by anti-cholinesterases which we have observed. Therefore a significant comparison of the relative activities of choline,

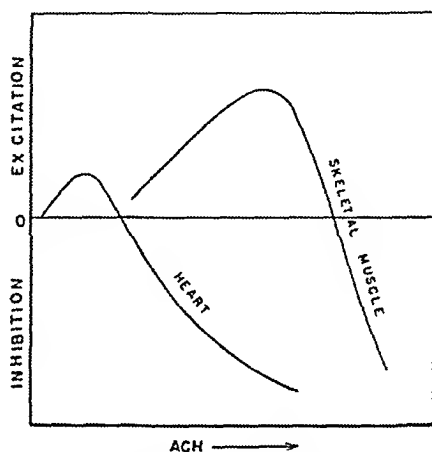


FIG 2 SCHEME SHOWING PROBABLE EXCITATION AND INHIBITION EFFECTS OF ACh ON VERTEBRATE HEART AND SKELETAL MUSCLE

choline derivatives and related compounds in the absence of cholinesterase inhibitors may be made. Such a comparison is shown in Fig 3. It may be seen that esters of both choline and betaine are far more active than the parent compounds and that the known differences in the resistance of the esters to esterase hydrolysis are unimportant in determining relative activity.

When ethyl radicals are substituted for methyl in the onium group of choline or Ach, activity is lost and concentrations as great as $2 \times 10^{-4}M$ are without effect. This directs attention to this portion of the molecule, and the action of quaternary ammonium compounds on the Venus heart becomes of interest. Tetra-methyl ammonium has an Ach-like action as may be seen in Fig 4, although its action is weak compared with Ach. Tetra-ethyl, tetra-propyl and tri-ethyl octyl

RELATIVE MOLAR QUANTITIES PRODUCING AN EFFECT EQUIVALENT TO MOLAR QUANTITY OF ACh
EQUAL TO 1

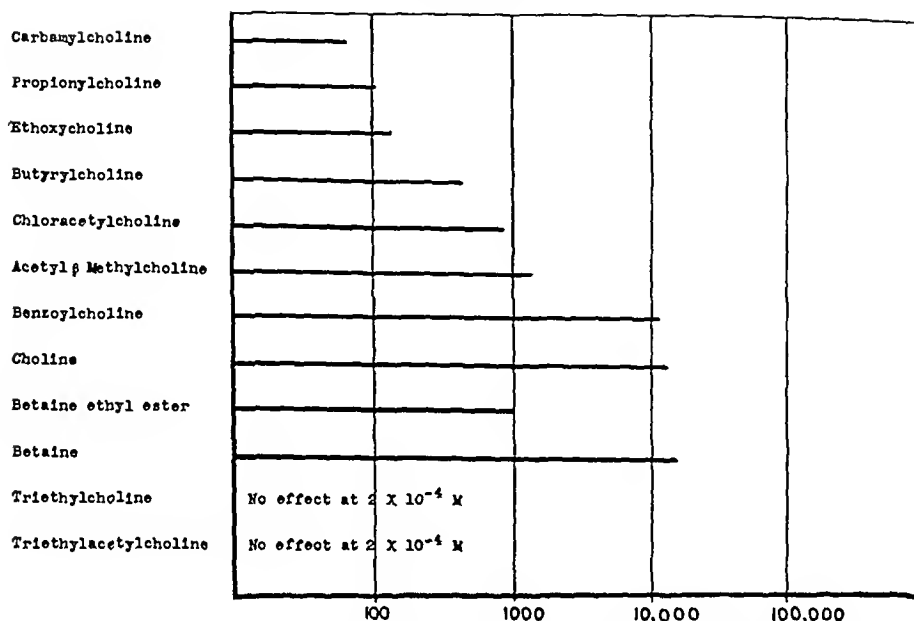


FIG 3 RELATIVE MOLAR QUANTITIES OF CERTAIN CHOLINE DERIVATIVES AND RELATED COMPOUNDS REQUIRED TO PRODUCE A DEGREE OF INHIBITION OF THE VENUS HEART EQUIVALENT TO THAT PRODUCED BY A MOLAR CONCENTRATION OF ACETYLCHOLINE TAKEN AS ONE

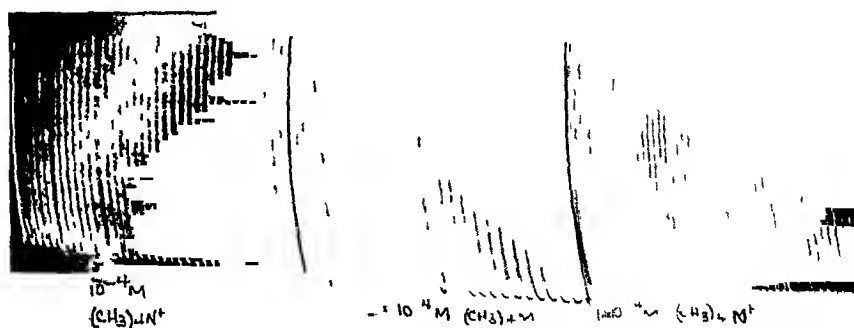


FIG 4 ACTION OF TETRA-METHYL AMMONIUM CHLORIDE ON THE ISOLATED HEART OF VENUS MERCENARIA AT THREE DIFFERENT CONCENTRATIONS (10^{-4} M, 1.2×10^{-4} M, 1.1×10^{-4} M)

ammonium ions, on the other hand, have no inhibitory action on the Venus heart at the highest concentrations tried (5×10^{-2} M). While tetramethyl ammonium sums with ACh, the other quaternary am-

moniums which we have tried are effective blocking agents—their effectiveness increasing with the size of the radical (Fig 5) Thus the pentavalent nitrogen with its three methyl groups becomes, in certain respects, the most significant portion of the Ach molecule

The rapidity with which Ach in small amounts acts on the Venus heart suggests that it is acting as a “trigger” to set off a reaction or chain of reactions The specificity of the $(\text{CH}_3)_3\text{N}$ group suggests that

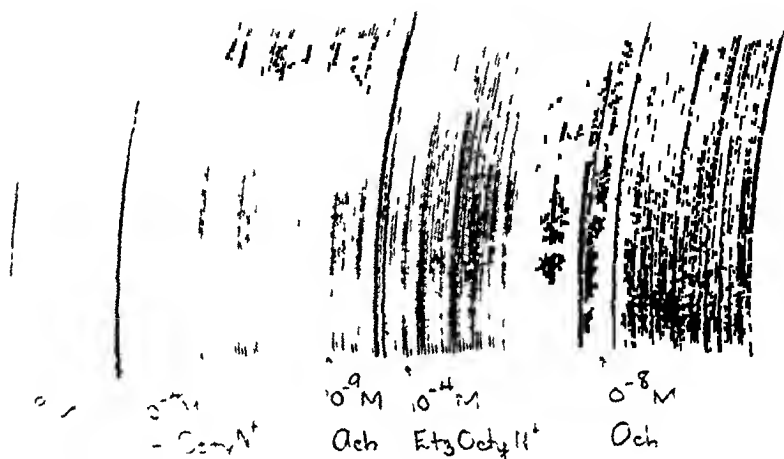


FIG 5 RECORD SHOWING THE EXCITATORY ACTION AND ACH-BLOCKING EFFECT OF TRIETHYL-OCTYL-AMMONIUM CHLORIDE ON THE ISOLATED HEART OF VENUS MERCENARIA

size and configuration are of the greatest importance The rapid recovery of the heart after washing and the rapid blocking action of quaternary ammonium ions other than tetra-methyl ammonium, when present with Ach in the medium surrounding the heart, suggest that Ach is acting at or near the surface of the smooth muscle membrane Evidence for surface action in the vertebrate heart is seen in the results of Cook (1926), who found that methylene blue effectively blocks the action of Ach on the isolated frog's heart *only when it is present in the fluid around the cells* If hearts were allowed to become deeply stained with methylene blue and then washed before adding Ach there was no antagonism, even though the cells retained their deep blue color

Cook suggested that this antagonism between methylene blue and Ach is due to the methylene blue producing some freely reversible surface action on the cells. The possibilities provided by methylene blue in physiological studies on the nervous system are not always fully appreciated (for a brief summary of its pharmacological action see Bernheim, 1942)

Although the action of Ach on the Venus heart as well as on vertebrate effectors, such as skeletal muscle, may be duplicated by potassium and electrical stimulation, there are certain indications that the effects of these latter agents are direct while that of Ach is by an indirect route. In other words, although externally applied K^+ and Ach, as well as a depolarizing current, appear to produce the same end result, they may do so in different ways. One is led, therefore, to speculation concerning the manner in which Ach might affect the excitability of cells, in the hope that such speculation will lead to newer approaches to the problem of the mode of action of Ach.

Certain investigators, notably Langley (1906) and Clark (1937), have postulated that certain drugs produce their effects on cells by reacting with a "receptive substance". This will be assumed in the hypothesis to follow which, for the sake of brevity, will be presented without an attempt to fully justify the line of reasoning.

- (1) Ach reacts with some constituent of the cell surface ("receptive substance")
- (2) This constituent of the surface is a protein or lipo-protein
- (3) The protein or lipo-protein is an enzyme requiring Ach for its activation, therefore Ach acts as a coenzyme
- (4) The enzyme reacts with a substrate in the cell membrane
- (5) This substrate when split releases nonpolar-polar anions (cf Hober, 1946) whose changing orientation might then account for observed polarity and permeability changes in the cell membrane and "excitation" of the cell
- (6) Ach is freed from the enzyme complex, split by cholinesterase or returned to inactive precursor, and energy-yielding reactions reconstitute the substrate restoring the cell membrane to its resting condition

Most existing hypotheses attempting to account for the mechanism of action of Ach are of a simpler nature. There is no virtue in a more

complex theory unless it is to direct attention to possibilities which might otherwise be overlooked. If all aspects of the above-outlined, possible mode of action of Ach were discarded, except the suggestion that Ach acts as a coenzyme, the present purpose would be served, this purpose being to direct attention to the unique properties which qualify Ach as a regulator of enzyme reactions which must start and stop within very brief periods of time. Of these properties, the readiness with which Ach may be hydrolyzed and thereby removed from the scene of action is perhaps the most important.

In testing the above hypothesis, one might begin with attempts to learn more concerning the nature and distribution of the "receptive substance"² We have begun efforts in this direction and, at present, a clue given by Couteaux (1947) is being followed. Couteaux has found that Janus green B used as a supra-vital stain selectively stains a group of oriented bodies lying in the sarcoplasm just under the sarcolemma and adjacent to nerve ending in the motor end plates of skeletal muscle. Janus green B also stains faintly the sarcolemma of the muscle fiber. We find that Janus green B stains muscle fibers in the atrial regions of the Venus heart where nerves end in the heart. This staining with Janus green would be of special interest only if it could be shown that the dye had Ach-like properties and might, therefore, combine with a substance with which Ach normally reacts. In preliminary experiments it has been found that Janus green does have Ach-like properties. The Venus heart is inhibited by Janus green as by Ach (Fig. 6). (Little is known concerning the pharmacological action of Janus green in vertebrates, but one may refer to Ettinger (1932) for information concerning its constrictor action on certain blood vessels of the intact mammal.) It also acts as an inhibitor of cholinesterase, as does methylene blue. This might complicate its use as a possible indicator of the distribution of "receptive substance," but at the same time may, when used with more specific anticholinesterases and Ach-blocking agents, provide valuable information. For example it might be possible to alter the staining properties of the "bâtonnets" of Couteaux by previous treatment with appropriate

² That more than one such substance (enzyme) is involved in Ach action seems evident from such well-known observations as the differences in blocking action of curare and atropine at different points within the body.

agents, thereby giving a clearer indication of their nature. This we plan to try.

This approach to the problem of the location and identification of the "receptive substance" is but one of many which are possible. The identification and probably the isolation of the substance or substances with which Ach reacts will be a necessary step in determining the precise mode of action of Ach. It may be a slow and difficult process.

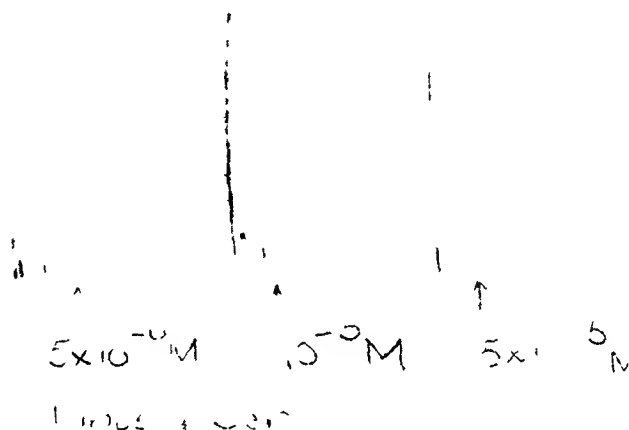


FIG. 6. RECORD SHOWING THE ACH-LIKE ACTION OF JANUS GREEN B ON THE ISOLATED HEART OF VENUS MERCENARIA.

While it is going on, one should not lose sight of the broad problem of the possible rôle of Ach as one of the regulators of cellular metabolism and of growth.

SUMMARY

In this paper an attempt has been made to encourage a broader outlook on the problem of the physiology of acetylcholine (Ach). Evidence suggesting that Ach may play a rôle in the regulation of cellular metabolism and growth has been presented. It was the intent to imply that this more general rôle of Ach accounts for its wide distribu-

tion in plants and animals From recent uncompleted studies, ideas have come which have given rise to a new view of Ach action, namely that it may be acting as a coenzyme to regulate the activity of an enzyme(s) ("receptive substance") located in or near the cell membrane The rôle of this enzyme is to alter the excitability of the cell through processes leading to changes in membrane polarity and permeability The instability of Ach makes it a possible regulator of an enzyme process that must be quickly started and stopped

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DISCUSSION

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Dr. Welsh's interesting speculations deserve an analysis on the basis of appropriate facts. Unfortunately, these are not available in sufficient number and so the discussion, at this stage, also must be largely on a speculative basis.

However, a number of considerations lead, rather forcibly, to the idea that such extremely highly active drugs as acetylcholine (ACh) and its counterpart adrenaline—both natural to the body—act on selected, small, strategic portions of the cell surface.

1 The intracellular administration of ACh in striated muscle (1) (also of HCN and H_2S (2) and narcotics (3) in amoeba) does not produce the characteristic actions which are readily elicited by application to the external surface of the cell. Cook's experiments, described by Dr. Welsh, likewise show that the atropine-like action of methylene blue is exerted only when the dye is at the external surface of the cells of the frog heart. The surface site of fixation or adsorption¹ of drugs indicates that ACh would not be expected to penetrate the cell to elicit its effects.

2 The minimal effective dose if spread out in a monomolecular layer occupies only a minute fraction of the cell surface. Clark (4) has calculated that only 1/6000 of the surface can be covered by a minimal effective dose of ACh inhibiting the frog heart.

3 The characteristic exquisite sensitivity of tissues such as the heart is apparently only acquired after the cells have received their innervation. The most recent clear-cut experiments in this field have been done by Armstrong (5) on the Fundulus heart. The nerves must induce a permanent change in the effector cells since the drug sensitivity persists (it is, in fact, enhanced) after the nerves have been caused to degenerate. From Langley (6) on, experimenters have shown that in the case of muscles with a 1:1 nerve to muscle fiber relation the sensitivity is confined to the end plate region.

In trying to supply a mechanism by which a substance acts with

¹ Clark (4) has shown that the reaction closely follows the Langmuir adsorption formulae

great rapidity, in trace amounts, at strategic points on an interface, the cell membrane, the possibility that an enzyme or its prosthetic group constitutes the receptor is a very reasonable one, but one that still awaits proof. Enzymes are ordinarily concentrated at and exert optimum activity at interfaces, such as the cell membrane. To call ACh a coenzyme is to do no more than to suggest that it participates in a reaction involving enzymes, and it is certainly true that reactions of this rapidity—including fixation and subsequent action—are almost certainly catalyzed and regulated by enzymes somewhere along the line to completion. However, in readily admitting the likelihood of enzyme availability for reaction with drugs, we must realize that they are not necessarily the point of attack when drugs influence the process variously viewed as depolarization, permeability change, reorientation of cell membrane constituents or the alteration of the state of protein chains abutting on the cell surface.

The fact that the receptor must compete with cholinesterase for ACh, and also for atropine and curare (7) and other drugs in the group I once called cholinotropic (8), would appear to be an indication that the receptor has at least a similar prosthetic group in common with the hydrolyzing enzyme and with the drugs mentioned. We might visualize the receptor as possessing a sort of master or, better, a skeleton key for the entire group.

It must be readily apparent that, though we are concerned with the mode of action of acetylcholine, we have really not gotten beyond the question of the site and manner of its fixation, only subsequent to which the reaction can occur. Whether the resulting change is an excitation or increase, or an inhibition or decrease in some aspect of cell function, we can, if we wish, quite profitably think of most drugs as becoming fixed to and always *exciting* or *activating* specific receptors, which in turn trigger off excitatory or inhibitory processes within the cell.

It is necessary to distinguish between true inhibition and the depression, often postexcitatory, accompanying larger or excessive doses. The latter² has often been compared to fatigue and resembles it at least

² Brown, Dale and Feldberg (9) have postulated in the case of striated muscle that the paralytic action of large doses of ACh is not confined to the end plate region. Brown and Feldberg (10) likewise picture a more diffuse action for the depressant effect in the superior cervical sympathetic ganglion of the cat.

in that, as a rule, it is less readily reversible than the primary inhibition. I do not feel that such depression is a true inhibition, in the same sense as the specific primary effect of small doses. True inhibition produced by drugs is not preceded by excitation in the vertebrates.

The excitatory effect of ACh in the atropinized mammalian heart has been found by Hoffman et al (11) and by McNamara, Krop, and McKay (12) to be due to the release of a sympathomimetic substance whose action can be blocked by ergotoxine. McDowall (13) has also shown that the stimulating effect could be abolished by ergotoxine and by still larger doses of atropine. These facts are best explained by assuming a cholinergic synapse—therefore activated by ACh and blocked by atropine—whose postsynaptic fibers release a sympathomimetic substance. This description would fit best a sympathetic ganglion located in the heart itself, and the action of atropine is similar to the reversal it induces in ACh vasodepression. This reasoning is not weakened by McDowall's demonstration of the effect without the use of atropine in the mammalian ventricle after cutting the A-V bundle, since, as McDowall himself points out, the mammalian ventricle receives no vagus innervation. It would therefore not be responsive to acetylcholine but would be excited by the cholinergically released sympathomimetic substance. The effect ordinarily masked in the non-atropinized heart is thus exposed by isolating the ventricle.

It is rather difficult with ordinary pharmacological techniques to record the immediate or very early course of drug action. I therefore wish to show you such a record for the synaptic inhibiting action of the functional counterpart and antagonist of ACh—adrenaline. Here also Burn (14) has brought up the question of an exciting action preceding the synaptic inhibition of sympathetic ganglia described by Marrazzi (15). Fig. 1 illustrates the technique and Fig. 2 shows the graph of continuously recorded results. It is evident that the first and only action is an inhibition indicated by the depression of the two postsynaptic spikes. Continuous visual observation of the oscillograph throughout the experiment likewise showed no evidence of preliminary stimulation.

If a preliminary stimulating effect in the heart of this mollusc (*Venus mercenaria*) were confirmed, it would only raise again the question of the nature of the ACh action in this organ in which, contrary to find-

ings elsewhere, the very characteristic and, so to speak, diagnostic effect of atropine is missing (Prosser, 16) and the effect of cholinesterase on the intensity of action is negligible

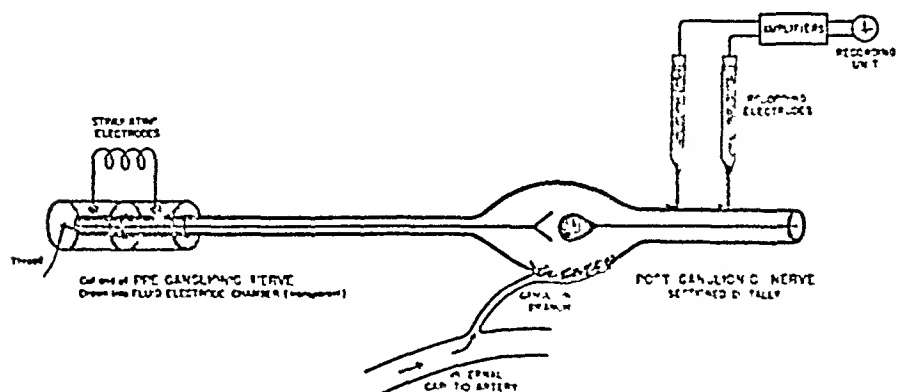


FIG 1 SCHEME OF STIMULATING AND RECORDING SET UP
SUPERIOR CERVICAL SYMPATHETIC GANGLION WITH INTACT BLOOD SUPPLY
From J Neurophysiol, 10 167, 1917 (Fig 1)

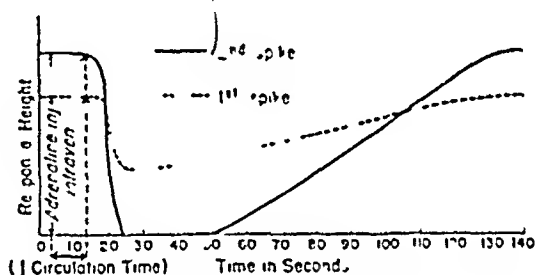


FIG 2 PLOT OF CONTINUOUS RECORD SHOWING DEVELOPMENT AND COURSE OF
GANGLIONIC INHIBITION BY ADRENALIN
From J Neurophysiol, 10 167, 1917 (Fig 4)

Although the distinction between primary inhibition and secondary depression is important, I do not believe it is crucial to further consideration of Dr Welsh's arguments and results

The Onium cation, which "must be regarded as the first pharmacodynamic group to be discovered" (17) again emerges in this preparation as the important group in quaternary ammonium bases. Dr Welsh makes an effective point in correlating the rapid wash out and the rapid blocking action with a surface locus of activity

Experience shows that a demand for a rigid or strict correspondence of chemical structure between compounds with similar or related effects, e g ACh, the various anticholinesterases, atropine, methylene blue, would set up unnecessary difficulties. On the other hand, the experience of enzyme chemists shows that coarse relations or rather relations restricted to a small portion of the molecule are not unusual. It follows from some of these ideas that stains delineating well the *functional* motor end plate would be expected to have some ACh-like, anticholinesterase and even atropine-like or blocking effects.

One of the most exciting parts of Dr. Welsh's report deals with his plans for studying the receptor substance by utilizing staining techniques. Some of the obvious difficulties which will have to be overcome by such an approach are illustrated by the effects of curare, which in strong solutions will cause motor end plates to become opaque (18), or more aurophilic (19), but which, according to others (20), will not alter the appearance of the end plates in doses adequate to curarize.

The remarks so far have been limited to processes taking place at neuro-effector junctions and in autonomic ganglia. A brief look at the more uncertain ground of the central nervous system is in order.

There is great difficulty in relating the ACh content of tissue to function, if indeed there is any direct relation. We may recall that Brown and Feldberg (10), in their study of the ACh metabolism of a sympathetic ganglion, concluded that they "could not assign a suitable rôle to the acetylcholine or the choline obtainable from a ganglion by extraction." A complicating factor is that, so far, ACh is found practically only in efferent fibers and no data is available on the ratio of efferent to afferent cells and fibers in various parts of the nervous system nor, for that matter, on the ratio of excitable to supporting tissue. Furthermore, as McIntosh (21) points out, ACh is, on the one hand, virtually absent from the pyramidal tracts and, on the other hand, is present in great abundance in non-nervous tissue like the spleen and placenta, which have no obvious need for special communication facilities. Accordingly, McIntosh regards as better founded deductions based on the absence rather than on the presence of extractable ACh. Since Dr. Welsh's results are on the basis of water extractable ACh only (no protein denaturants used), they are perhaps not exactly comparable to those just quoted. Nevertheless, similar difficulties must operate

Himwich et al (22) have established that in general the metabolic rate increases as the neuraxis is ascended, and that the immature rat brain has a lower rate and is more resistant to anoxia than the adult. The corollary seems to hold that resistance to anoxia parallels decreasing metabolic rate. If, then, there is a relation between ACh content and function, could not as good a case be made out for the idea that larger stores of ACh (larger production with lower cholinesterase activity in the infant rat brain) are associated with regions of lower metabolism and consequently greater resistance to anoxia?

Need the accelerating effect of ACh on salivary gland slices mean any more than that the secretory cells are activated and oxidation secondarily follows suit? Lipton (23) failed to find an increase due to ACh in rat brain, while Shaffer, Chang and Gerard (24) observed a decrease in peripheral nerve.

Denervated tissues undergo atrophy of disuse unless they are capable of autonomous activity. Since ACh and related drugs activate denervated tissues and otherwise control cholinergically innervated tissues, need we look for a trophic process separate from transmission and conduction to explain Dr. Welsh's striking preliminary results on planaria and those of Ward and Kennard on monkeys?

Dr. Welsh has admirably explored many stimulating possibilities and signaled out deficits in our knowledge. We also have the feeling along with Dr. Welsh that, given an extensively studied field, further progress is often touched off by new or different techniques. We have, therefore, made preliminary excursions in measuring the electric field (dipole moment¹) of drugs to study its possible determining influence on their orientation to the sensitive areas of the cell surface, the receptors, and we are initiating the further study of drug action on suitable films to analyze in greater detail their surface or interface activity, and, more particularly, the associating forces between drugs and active patch or receptive substance.

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V THE EFFECT OF ANTICHOLINESTERASES AND RELATED SUBSTANCES ON NERVOUS ACTIVITY IN THE COCKROACH^{1 2}

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INTRODUCTION

While the specific objective of this paper is to discuss the physiology and pharmacology of the sixth abdominal ganglion of the cockroach it is hoped that attention can be drawn to the potentialities of insects as material for basic research in physiology. To some, the small size of insects may appear to handicap their possibilities in this direction. However, experience has shown that where electrophysiological methods can be used, small size of the material may be advantageous, since the number of neurones to be encountered is much less. The suitability of insects for certain types of research may be judged from the fact that in a relatively modest research program at Tufts College, the common cockroach, *Periplaneta americana*, has provided two or three different types of ganglion preparations (9-12), a giant fiber preparation, a sensory preparation consisting of fibers from specialized mechanoreceptors (8, 11), and a neuromuscular preparation. Some of these preparations have not been investigated fully, and cannot be discussed at this time. The sixth abdominal ganglion has been studied in some detail, and will serve to illustrate the research possibilities of insects.

THE PREPARATION

The abdominal portion of the ventral nerve cord of the cockroach terminates in a ganglion about 0.9 mm. in length. This is the sixth

¹ The work described in this paper was done under a contract between The Medical Division, Chemical Corps, U. S. Army, and Tufts College. Under the terms of this contract, the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the author.

² The author wishes to express his gratitude to Mrs. Nancy K. Kennedy, and Miss Elizabeth A. Weiant, who carried out most of the observations on anticholinesterase action.

abdominal ganglion (fig 1), which serves as a sensory and motor center for the terminal abdominal segments. The major sensory supply to the ganglion consists of a pair of cercal nerves from each of the cerci. The cercal nerves appear to be entirely sensory, and contain a large

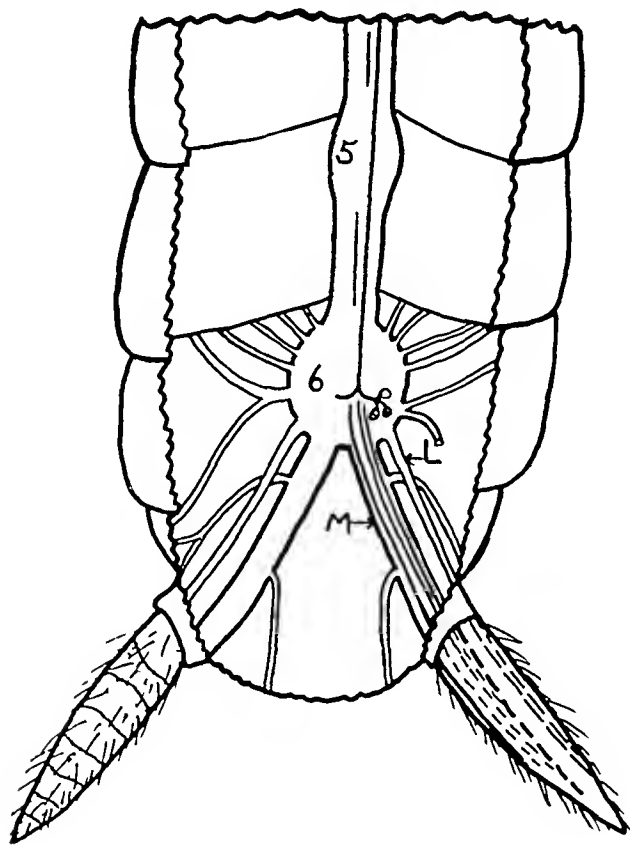


FIG 1 DIAGRAM OF THE TERMINAL PORTION OF THE COCKROACH NERVE CORD

The lateral (L) and medial (M) cercal nerves enter the hemocoel from the base of the cerci, and pass to the sixth abdominal ganglion. One giant fiber is shown ascending the ventral cord. The neurone relations shown are approximate.

number of afferent fibers from sense cells located at the bases of fine hair sensilla which invest the cerci. Pumphrey and Rawdon-Smith (9) have shown that the hair sensilla constitute a primitive auditory organ, or wind gauge, mechanically responding to air movements ranging from gentle air currents up to air-borne vibrations in the audible

range. On entering the sixth abdominal ganglion, fibers from the sense cells of the hair sensilla synapse with six to ten large fibers ranging from 10 to 50 microns in diameter. These so-called giant fibers can readily be identified in sections of the abdominal cord, which they ascend without synaptic interruption. On entering the thoracic ganglia the giant fibers form connections with motor centers concerned in the evasion reflex. Physiological evidence indicates that many afferent fibers from the cercal nerves synapse with each ascending giant, and that the majority of afferent fibers form only homolateral connections. Information on anatomical relations within the ganglion is very meagre, though there are indications that the giant fibers originate as multicellular neurones, and that the afferent fibers form a complex net-work around the fibers near their origin from four or five cell bodies in the cortex of the ganglion.

METHODS

Impulse conduction through the ganglion is studied by electrical stimulation of the cercal nerves on one side. A monophasic square pulse of 300 microseconds duration is applied through fine tapered silver electrodes in contact with the nerve as it emerges from the base of the cercus. The post-synaptic response is recorded either from the anterior part of the ganglion, or from giant fibers in the homolateral connective of the nerve-cord. Amplifying equipment consists of two channels of Grass P3 capacity couple amplification. The oscilloscope is a double-gun 5SP11 tube installed in a modified 247 DuMont oscilloscope (Bullock, 1).

IMPULSE TRANSMISSION THROUGH THE SIXTH ABDOMINAL GANGLION

The nature of transmission through the ganglion is illustrated by figure 2. The upper trace in each record was obtained from electrodes under the cercal nerve at its entrance into the ganglion, and the potential change is due to the arriving pre-synaptic volley. The lower trace was obtained from the point where the nerve-cord arises from the anterior end of the ganglion, and records the departure of the post-synaptic volley in the giant fibers. The stimulus was applied to the cercal nerve at the base of the cercus at a frequency of 1 per second.

All records in figure 2 were obtained from the same preparation and differ only in the strength of the stimulus applied

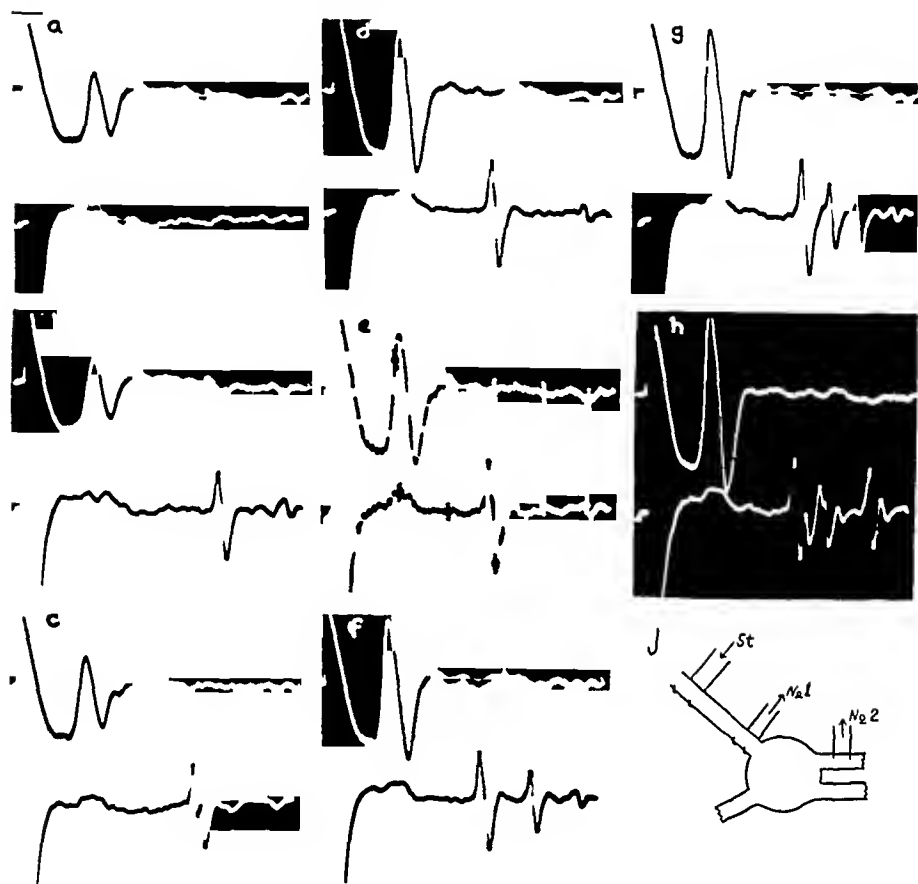


FIG 2 PRE- AND POST-SYNAPTIC RESPONSES IN THE SIXTH ABDOMINAL GANGLION OF THE COCKROACH

Approximate electrode positions are shown in J. The upper trace in each record consists of the compound spike potential in cercal fibers entering the ganglion. The lower trace records spike potentials in giant fibers leaving the ganglion. The stimulus applied to the cercal nerve was progressively increased through the series. For full explanation, see text. Stimulus frequency, 1/sec. Time is given by 10 kilocycle modulation of the beam intensity in E. Every tenth cycle is marked by a vertical line.

A measure of the synaptic stimulus is the size of the pre-synaptic volley, which increases with the stimulus throughout the series. Direct observation of the pre-synaptic volley on the oscilloscope shows that

its growth is practically stepless as the stimulus is increased, and it appears to be compounded of a considerable number of small spike potentials. In figure 2a the pre-synaptic volley is considerable, though there is insufficient spatial summation to fire any of the post-synaptic giant fibers. A slight increase in the presynaptic volley reaches the threshold of a single post-synaptic fiber (2b), and a single spike potential appears abruptly in the lower trace. Further increments in the pre-synaptic volley (c, d, e) cause a slight increase in the post-synaptic spike, due presumably to the addition of smaller spikes, though the most noticeable change is a shortening in the conduction time for the post-synaptic response which is in no way matched in the pre-synaptic volley. This shortening of the trans-synaptic conduction time (utilization time) may amount to as much as 0.8 millisecond. A further increase in the size of the pre-synaptic volley (f, g) causes the appearance of two more post-synaptic spikes. The fibers which contribute the latter appear to respond only at maximum utilization time, since a further increase in the pre-synaptic volley (h) causes them to approach and eventually to sum with the original spike. The pre-synaptic volley at this point reaches the threshold of a fourth giant fiber.

Measurement of the synaptic delay is being attempted by two methods, analysis of potentials recorded from the surface of the ganglion, and analysis of cord potentials initiated by direct stimulation of the ganglion. Results are incomplete, but the minimum synaptic delay exclusive of the utilization time appears to occupy 1.1 milliseconds.

CHEMICAL RESPONSES OF THE SIXTH ABDOMINAL GANGLION

Provided that it is not exposed to prolonged periods of rapid stimulation, the ganglionic preparation described above is remarkably stable. Many preparations have been stimulated intermittently for six to eight hours with little or no change in threshold. The saline used to bathe the preparation and to apply the agents has the following composition, NaCl 9.0 gm, CaCl_2 0.2 gm, KCl 0.2 gm, H_2O 1000 ml. Ten ml isotonic phosphate buffer is added to buffer the saline to pH 7.2. It has been shown that the cockroach nervous system will tolerate wide variations in calcium and potassium (10).

Anticholinesterases The effects of physostigmine, disopropyl

fluorophosphate, and hexaethyl tetraphosphate on synaptic transmission in the sixth abdominal ganglion have been described elsewhere (12, 13) The characteristic effect of these substances is to facilitate

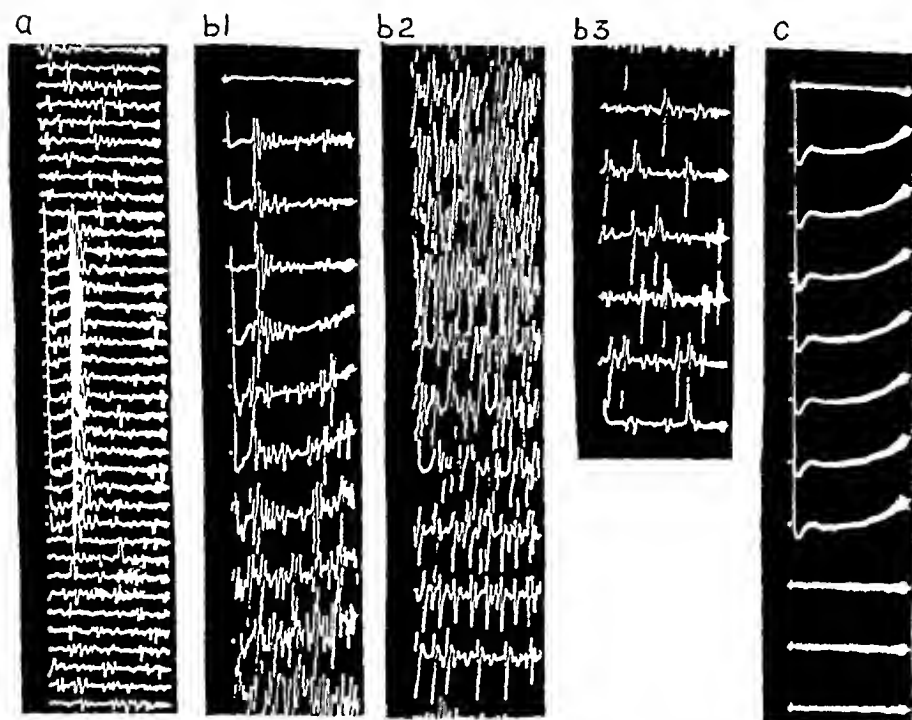


FIG 3 THE EFFECT OF DFP ON TRANSMISSION THROUGH THE SIXTH ABDOMINAL GANGLION

A Normal ganglion, 17 stimuli (10/sec) given near middle of trace. Stimulus artifact appears at the beginning of each sweep. B Ganglion treated with 5×10^{-5} M DFP, 9 stimuli (3/sec) begun on second sweep. After a few normal responses, after-discharge begins and continues after stimulus is turned off (second from last sweep, B1). It continues for some time (B2 and B3) and is followed by a block (C).

synaptic transmission at low concentrations, blocking it at higher concentrations. Though the post-synaptic fibers normally respond with a single spike to each pre-synaptic volley, and are able to follow the latter up to frequencies of 400 per second, fifteen minutes after direct application of 5×10^{-5} M DFP, a brief series of afferent stimuli elicit a post-synaptic after-discharge which may last for 15 to 20 seconds (figure 3b). The after-discharge may be followed by a brief

period of normal synaptic transmission, or more usually by a synaptic block (fig 3c) The block may last for one to five minutes, when a pre-synaptic volley may cause another after-discharge Synaptic conduction remains in an extremely unstable state, and spontaneous or pre-synaptically induced after-discharges may alternate with block for several hours In no case was it possible to bring a DFP-treated preparation back to normal by washing with saline, though periods of prolonged block could be broken in this way, and the preparation brought back to the after-discharge condition Higher concentrations (up to 10^{-3} M) of DFP caused little additional effect, though the periods of block appeared to become longer

Hexaethyl tetraphosphate and tetraethyl pyrophosphate have very similar actions, both being more potent than DFP A solution of 3×10^{-7} HETP or TEPP will bring about the condition in which an afferent volley elicits an after-discharge, within 10 to 15 minutes Higher concentrations of these agents have a tendency to produce continuous synaptic block, while washing causes no return to normal, though the block may give place to the after-discharge condition

Physostigmine in 5×10^{-5} M solution has a similar effect though the after-discharge produced is very brief, and the blocking tendency is greater Prostigmine in a similar concentration causes only a synaptic block The effects of physostigmine and prostigmine can be removed by washing with saline

The effects of these substances are summarized in table 1 Included in this table are data from Chadwick and Hill (2), who determined the percent cholinesterase inhibition in the roach cord in the presence of a series of concentrations of DFP, HETP, and physostigmine It will be seen that there is a striking correlation between the concentration of each agent capable of inhibiting 93 to 96 percent of the cord cholinesterase, and the concentration just capable of producing the after-discharge The conclusion is inescapable that normal synaptic function in this ganglion is dependent on the presence of a certain level of cholinesterase

In contrast, transmission along giant fibers of the ventral cord is completely unaffected by these agents in the concentrations used above Since the giant fibers in the abdominal cord are uninterrupted by synapses, it is possible to study axonic transmission by stimulating the

cord just above the sixth abdominal ganglion, while giant fiber potentials are recorded from a higher level. The axonic effects of DFP and HETP were studied intensively, but no significant change in axonic conduction was noted until 3×10^{-3} M HETP or 6×10^{-2} M DFP were applied (table 1). At these concentrations there was an immediate conduction block which could be removed within a few minutes by washing with saline. A similar effect was first described by Crescitelli, Koelle and Gilman (4).

Since both DFP and HETP hydrolyse in aqueous solution to produce free acid, it was thought that hydrogen ions might be responsible for

TABLE 1
Summary of anticholinesterase action on the roach nerve-cord

AGENT	NEGATIVE LOG MOLAR CONCENTRATION CAUSING			
	Percent ChE Inhibition (in parentheses) After Chadwick and Hill	Synaptic after-discharge on afferent stimulus	Synaptic block	Axonic block
HETP	6.3 (94%)	6.5	6.5-6.0	2.5*
TEPP	n.t.	6.5	6.5-6.0	n.t.
DFP	4.3 (93%)	4.3	3.0	1.2
Phy. sostigmine	4.3 (96%)	4.3	4.3-4.0	3.0
Prostigmine	n.t.	none	4.3	n.t.

n.t., not tested

* No axone block produced when neutralized

the axone block produced. The preparation of concentration-pH curves for DFP and HETP in insect saline showed that at synaptically active concentrations (5×10^{-5} M and 3×10^{-7} M respectively) there was insufficient free acid produced to alter the pH of the buffered saline. At the concentrations producing axonic block (6×10^{-2} M and 3×10^{-3} M respectively), both agents in insect saline had a pH of 2.0 to 2.3. Neutralization with isotonic Na_2HPO_4 completely removed the axone-blocking ability of HETP without altering its action on synapses. As a further check, saline was acidified by the addition of phosphoric acid and applied to axones. When the solution had a pH of 2.0 to 2.5 an immediate and reversible axone block was produced, which appeared to be identical with that caused by unneutralized

HETP Thus, the axone-blocking ability of hexaethyl tetraphosphate, the most active anticholinesterase and synaptic agent studied, appears to be due only to acid produced on hydrolysis. Neutralization of DFP in a similar manner failed to eliminate its ability to block axones, and its mode of action on the latter remains obscure.

Acetylcholine Stability of synaptic conduction across the sixth abdominal ganglion of the cockroach is dependent on the amount of cholinesterase present. The possibility that acetylcholine serves as synaptic mediator was investigated quite fully. Acetylcholine chloride (LaRoche) and bromide (Merck), acetyl-beta-methylcholine, and carbaminoylcholine were applied to the ganglion in concentrations up to 10^{-2} M, both alone, and preceded by physostigmine, DFP, and HETP. When applied alone acetylcholine and related substances clearly had no action on transmission through the ganglion. When applied after treatment with an anticholinesterase, effects were very difficult to observe. It was reported earlier (13) that acetylcholine would block conduction under these circumstances, though a prolonged series of tests (30 to 40) performed later makes this conclusion very doubtful. All the anticholinesterases produce a condition of such synaptic instability that the mere application of a drop of saline to the ganglion has been observed to initiate an after-discharge and subsequent block. It seems likely that the earlier results were due to this effect, and it can safely be said that acetylcholine adds little if anything to the change produced by the anticholinesterase. Acetylcholine and related compounds were also applied to ganglia previously treated with concentrations of anticholinesterases just insufficient to produce synaptic instability. No effect could be noted. A similar lack of action of acetylcholine on Crustacean synapses has recently been reported by Shallek and Wiersma (14).

Though acetylcholine appears to be without action on synaptic conduction, it has been demonstrated repeatedly that the roach cord contains considerable quantities of acetylcholine (3, 6, 15). Determinations in this laboratory, using the frog rectus abdominis as assay object, gave an average figure of 32 gamma acetylcholine per gram of cord (wet weight) in the presence of 10^{-5} physostigmine, and 38 gamma per gram in the presence of 10^{-5} HETP, figures which are in substantial agreement with earlier determinations. During the assays,

portions of the cord homogenates to be tested on the rectus were also applied to ganglia, but had no effect other than that which could be attributed to the anticholinesterase present

Atropine, Scopolamine, and Curare In vertebrates, these drugs are thought to block either the muscarinic or nicotinic effects of acetylcholine by rendering post-synaptic elements insensitive to the action of the mediator. All of them are notably inactive on insects, and failed to affect transmission through the sixth abdominal ganglion. They were applied in concentrations up to 10^{-2} , at which point atropine appeared to block transmission in one or two cases. Lower concentrations (10^{-3}) were without effect either on normal ganglia, or on those which had been treated previously with anticholinesterases. The complete lack of action of curare was clearly demonstrated in an experiment in which a 10^{-7} solution of curare completely blocked the response to 10^{-7} acetylcholine in a frog rectus abdominis muscle previously sensitized with anticholinesterase. A solution of 10^{-2} curare from the same sample was completely without effect when applied to a ganglion showing after-discharges resulting from the same concentration of anticholinesterase.

DISCUSSION

These observations may be briefly summarized

- 1 The effect of DFP, HETP, and physostigmine synaptic transmission in the sixth abdominal ganglion is closely correlated with their antiesterasic activity

- 2 This correlation does not receive an explanation from studies on the action of acetylcholine and related substances which appear to be without synaptic action

- 3 The lack of action of acetylcholine is correlated with the lack of action of acetylcholine-blocking substances, such as atropine, scopolamine, and curare

- 4 The action of anticholinesterases on axonic transmission in the insect ventral nerve cord appears to be unrelated to antiesterasic activity. The most powerful anticholinesterase, HETP, failed to block axones when neutralized, though this did not affect its synaptic action

The alternating synaptic facilitation and block produced by DFP

and HETP suggests that cholinesterase is intimately concerned with regulation of the effect produced by the pre-synaptic volley. There is no indication that conduction in pre-synaptic fibers is affected, though the volley arriving at the ganglion may actually become smaller owing to a slight rise in threshold in the sensory fibers produced by DFP. In spite of this, the synaptic effect produced by the arrival of a pre-synaptic volley after partial inhibition of cholinesterase may last for several seconds, whereas it normally lasts for less than 20 milliseconds. Following almost complete inhibition of the enzyme, the effect of an arriving pre-synaptic volley may last indefinitely, and a prolonged synaptic block is established. At threshold concentrations of the enzyme inhibitor, it can be shown that destruction of cholinesterase alone is not responsible for synaptic block, but that the arrival of a pre-synaptic volley is necessary. It is possible, though difficult, to prevent a DFP-treated ganglion from blocking for some time. This can be done only by reducing the possibility of all forms of stimulation to zero, and the ganglion must be deafferented. If this is not done synaptic instability reaches the point where the slamming of a distant door, or a slight movement near the preparation is sufficient to stimulate a few hair sensilla and initiate after-discharge and block. At higher concentrations of DFP, after-discharges appear to arise spontaneously, though they can still be precipitated by a brief pre-synaptic volley.

All these observations point to the persistence of a synaptic mediator substance in the absence of cholinesterase, and the picture is very similar to that described by Marrazzi and Jarvik (5) in mammalian autonomic ganglia. At this point the similarity ends, since acetylcholine and acetylcholine-blocking drugs appear to be without action. Only two explanations can be seen at the moment. Either acetylcholine, acetyl-beta-methylcholine, carbamoylcholine, curare, atropine and scopolamine are unable to penetrate to synapses within the ganglion, while DFP, HETP, TEPP, physostigmine, and prostigmine are manifestly able to do so, or the synaptic transmitter in this ganglion is not acetylcholine. There appears to be little evidence to justify the first explanation, which has also been proposed by Nachmansohn and Rothenberg (7) to account for the differential effects of DFP on axones and synapses. The difference between the group of compounds which

are synaptically effective and the group which is not is rather one of physiological action than of chemical structure. If differential penetration is the answer, evidence will be difficult to obtain, since the amount of a compound accumulating in the interior of an axone seems to provide little information about the amounts which reach the active surface of the axone, which is thought to play the primary part in conduction. The second explanation is equally lacking for evidence, though the important rôle of cholinesterase in synaptic conduction suggests that the transmitter may be chemically related to acetylcholine.

Though it has been demonstrated that cholinesterase plays an important part in synaptic conduction, there is no evidence that this is the case with axonic conduction in the ventral nerve-cord. The most effective anticholinesterase, HETP, blocks axones only because of the free acid produced on hydrolysis. Though DFP in 6×10^{-2} M solution continues to block axones after neutralization, it is interesting to note that 5×10^{-2} potassium chloride also blocks axonic conduction in the nerve-cord, but unlike the anticholinesterases, it exhibits no preferential action on synaptic conduction (10). A somewhat lower concentration of hydrogen ions (pH 2.0 to 2.3) causes a similar axonic block. It seems likely that many substances will block axones in this concentration, and this effect must be regarded as non-specific and possibly physical until it is demonstrated to be otherwise.

SUMMARY

1 The characteristics of transmission across synapses in the sixth abdominal ganglion of the cockroach are discussed, and it is recommended as an excellent preparation for the study of synaptic events.

2 It is shown that di-isopropyl fluorophosphate, hexaethyl tetraphosphate, tetraethyl pyrophosphate, physostigmine, and prostigmine produce various degrees of synaptic facilitation (after-discharge) and synaptic block. The threshold potency of these agents in affecting synaptic transmission is closely correlated with their anticholinesterase activity.

3 Acetylcholine, acetyl-beta-methylcholine, and carbinoylcholine appear to have no synaptic action. Likewise, acetylcholine-blocking

substances such as atropine, scopolamine, and curare are without effect on synaptic conduction in the cockroach

4 The action of anticholinesterases on conduction along axones in the ventral nerve-cord appears to be unrelated to their anticholinesterase properties. Conduction block in axones is obtained only with high concentrations of the agents, and the most effective anticholinesterase, HETP, fails to block axones when neutralized, though this treatment does not affect its synaptic action.

5 It is concluded that cholinesterase is of primary importance in conduction across insect synapses, and that the synaptic change which follows destruction of the enzyme suggests persistence of a synaptic mediator substance. There is no evidence that the synaptic mediator is acetylcholine.

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DISCUSSION

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In a mammalian nerve muscle preparation, eserine, neostigmine and DFP have several characteristic actions. The muscle action potential under these circumstances is converted into a short asynchronous salvo indicating a repetitive response of the muscle fibers to a single nerve stimulus. Furthermore, the excitation of eserinizd muscle by a train of motor nerve volleys, at a frequency greater than 6 per minute, results in a progressive depression of the twitch tension. Stimuli applied to the motor nerve at a rate of 50 per second cause a brief contraction, followed by relaxation of the muscle. That a depression of neuromuscular conduction results from the accumulation of a paralyzing concentration of acetylcholine is suggested by the observation that the enhanced responses of eserinizd skeletal muscles to low frequency single nerve volleys are abruptly depressed by the concurrent intra-arterial injection of a minute amount of acetylcholine. All of these phenomena can be reproduced in the human subject by injecting the anticholinesterase into the brachial artery and studying the function of the muscle abductor digiti quinti in response to stimulation of the ulnar nerve. It will be noted that the voltage of the muscle action potential in response to a maximal motor nerve volley is not altered by the presence of an anticholinesterase, just as the postsynaptic potential

described by Dr Roeder is not altered under these circumstances. How far the analogy between the repetitive discharge of the muscle, in response to stimulation, and the blocking effect following activity can be compared to the phenomena which Dr Roeder has demonstrated in the nervous system of the cockroach one can not say, but the general pattern seems somewhat similar. The greatest difficulty is afforded by the fact that, in contrast to the neuromuscular system, the post-ganglionic fibers of this cockroach preparation do not seem to be sensitive to stimulation by applied acetylcholine.

It seemed of interest in relation to these experiments of Dr Roeder's to review briefly the effects of di-isopropylfluorophosphate on the central nervous system in man. The daily intra-muscular injection of this substance in normal subjects, who are not receiving any other medication, usually results in the development of symptoms referable to the central nervous system including excessive dreaming, insomnia, restlessness, increased tension, emotional lability, subjective tremulousness, nightmares, headache, increased libido, giddiness, drowsiness, paraesthesia, mental confusion, visual hallucinations, and occasionally pains in the legs of sciatic distribution. It is of interest that these symptoms are not significantly affected by the administration of neostigmine, but are diminished to some degree by atropine. Electro-encephalograms taken during the period in which these symptoms are present show an increase in the potential size and in the frequency and irregularity of the rhythm. In many instances there is the appearance of abnormal waves similar to those seen in patients with grand mal epilepsy. These electro-encephalographic changes are promptly reversed by atropine and are not affected by neostigmine or curare. All that one can say is that in these experiments central nervous system effects have been produced by DFP, which to date is shown to have no other important action than its ability to destroy cholinesterase. The production of these central nervous system effects by a compound which has been shown to inhibit cholinesterase within the brain suggests that the acetylcholine cycle does play a positive but as yet undefined rôle in central neural function. The point which I wish to emphasize here is that in the human subject one is able to study the effects of this drug without the introduction of conflicting factors, and

in the normal human subject one is able to record the subjective alterations in normal nervous system function as well

I think it is also worth pointing out very briefly that certain additional technics are available by which the clinical neurophysiologist may be able to obtain more detailed knowledge of central nervous system function and to study in a more precise fashion the effects of various diseases and chemical agents on this function. Piper, in his monograph on *Electrophysiology of Human Muscle*, was the first to suggest that conduction velocity in a peripheral nerve may be measured by stimulating the nerve at two different sites, measuring the latencies of the muscular responses evoked by each shock, and dividing the latency difference by the distance between the points of stimulation. The following illustration is taken from his monograph published in 1914

Even with the relatively primitive electrophysiological technics available during the early part of this century, clinical physiologists became interested in their applications to the study of spinal cord reflex activity in human subjects. Hoffman was the first to demonstrate the feasibility of studies of this type. He first devised a method for recording the impact of a blow on the patellar tendon and in the same record the electromyogram from the quadratus muscle. He realized that the measurements were complicated by the characteristics of the sensory end organ, and going a step further found that by electrical stimulation of the mixed nerve he was able to excite a reflex response which could be recorded electrically. It is possible using this technic to stimulate with a current of just sufficient strength to excite only one motor unit in the muscle from which one is recording and also to set up a reflex response involving this same motor unit.

Dr. George Dawson found in studying a patient suffering from myoclonic seizures that electrical stimulation of a peripheral nerve produced an electrical potential detectable on the scalp. These changes in potential were detected over the hemisphere on the side opposite to that stimulated. They were located near to the midline when the lateral popliteal nerve in the leg was stimulated and more laterally when the ulnar nerve was stimulated in the arm. Extending these studies to healthy subjects, Dawson has been able to demonstrate that potential changes of cerebral origin, which probably arise in the central

and post-central cortex, may also be detected on the scalp following electrical stimulation of peripheral nerves. In the record shown in this slide taken from Dawson's paper which appeared recently, one can see the responses to stimulation of the left and right median nerves at the elbow and the left lateral popliteal nerve at the head of the fibula. The electrodes were applied over the surface markings of the sensory-motor area in the midline and 6 and 12 cm from it on either side. A stimulus to the left median nerve in A produced a potential change maximal near the electrode on the right side common to leads one and two, six cm from the midline. A stimulus to the right median nerve B produced a similar disturbance on the left side of the head. Stimulation of the left lateral popliteal nerve gave rise to a potential change nearer to the midline electrode than to any of the others. The latency of the response to stimulation in the leg was 36 plus or minus 2 m sec, about 14 m sec longer than when the median was stimulated.

One can see that, although the methods are still relatively crude and the potential contributions of the clinical neurophysiologist are still in the developmental phase, it is becoming possible to gather more and more reliable facts. The unique physiological experiments created by naturally occurring disease, combined with a study of the effects of various chemical agents on nervous system function in human beings, may uncover many stimulating leads. These may serve as a complement to the interesting and valuable studies which Dr Roeder has presented this afternoon. There are a few questions of a general nature which I would like to ask Dr Roeder.

- 1 Has any other pattern of stimulation been employed in these experiments?
- 2 Whether there are any threshold changes demonstrable following the application of acetylcholine, using presynaptic volleys just insufficient to stimulate any postsynaptic fibers?
- 3 What happens when the presynaptic nerves are cut and allowed to degenerate as far as the effect of DFP and acetylcholine application are concerned?

INDEX TO VOLUME LXXXIII

Pagination according to months

July, 1948, 1-118	
August, 1948, 119-185	
September, 1948, 187-278	
October, 1948, 279-365	
November, 1948, 367-462	
December, 1948, 463-603	
Acetylcholine, Symposium on the Physiology of	463
Acetylcholine as a Pharmacological Agent Koppányi, Theodore	532
Alloxan Diabetes, Antibody Formation in	326
Amino-Acids, Assimilation of	119
Anesthesia and Operation, The Cardiac Mechanism during in Patients with Con- genital Heart Disease and Cyanosis	237
Anoxia, Adaptation to in Congenital Heart Disease with Cyanosis	439
Antibody Formation in Alloxan Diabetes Payne, Torrence P B and Cruickshank, Alan H	326
Assimilation of Amino Acids, The	119
Bing, R J Physiological Studies in Congenital Heart Disease VI Adaptation to Anoxia in Congenital Heart Disease with Cyanosis	439
Bodian, David The Virus, the Nerve Cell, and Paralysis A Study of Experimental Polomyelitis in the Spinal Cord	1
Book Reviews	116, 181, 457
Books Received for Review	118, 462
Campbell, J A (See Bing, R J)	439
Cardiac Mechanism during Anesthesia and Operation in Patients with Congenital Heart Disease and Cyanosis Ziegler, Robert F	237
Carlner, Paul E (See Gay, Leslie N)	356
Cerebellopontile Recess, Tumors at the	187
Circulating Anticoagulant as a Cause of Hemorrhagic Diathesis in Man Conley, C Lockard, Rathbun, Howard K , Morse, William I , II, and Robinson, James E , Jr	288
Clinical Manifestations of the Severe Form of Diphtheria, The Fisher, A Murray and Cobb, Sidney	297
Coarctation of the Aorta, Renal Function Before and After Surgical Resection of	429
Cobb, Sidney (See Fisher, A Murray)	297
Comparative Study of Antihistamine Substances	
I Introduction and Dale Experiments Landau, S Walter and Gay, Leslie N	330
II Activity in Vivo Against Histamine Intoxication and Anaphylactic Shock of Guinea Pigs Landau, S Walter, Marriott, Henry J L , and Gay, Leslie N	343
III Clinical Observations Gay, Leslie N , Landau, S Walter, Carlner, Paul E , Davidson, N S , Furstenberg, Frank F , Herman, Nathan B , Nelson, William H , Parsons, John W , and Winkenwerder, Walter W	356

Concerning the Mode of Action of Acetylcholine	Welsh, John H	568
Congenital Heart Disease, Physiological Studies in		439
Congenital Heart Disease and Cyanosis, The Cardiac Mechanism during Anesthesia and Operation in Patients with		237
Conley, C Lockard	Circulating Anticoagulant as a Cause of Hemorrhagic Diathesis in Man	288
Crowe, Samuel J	(See Proctor, Donald F)	383
Cruickshank, Alan H	(See Payne, Torrence P B)	326
Cultural Differentiation of Paracolon Bacilli, The	Schaub, Isabelle G	367
Darrow, Chester W	(See Koppanyi, Theodore)	561
Davidson, N S	(See Gay, Leslie N)	356
de Majo, Salvador F	(See Nyda, Morton J)	279
de N6, R Lorente	Quaternary Ammonium Ions and Sodium Ions in Nerve Physiology	497
Diabetes, Alloxan, Antibody Formation in		326
Differential Diagnosis of Tumors at the Cerebellopontile Recess	Revilla, Antonio Gonzalez	187
Diphtheria, The Clinical Manifestations of the Severe Form of		297
Effect of Anticholinesterases and Related Substances on Nervous Activity in the Cockroach	Roeder, Kenneth D	587
Effect of Ovariectomy and Physiologic Doses of Estradiol upon Body Weight, Linear Growth, and Fat Content of the Female Albino Rat	Nyda, Morton J, de Majo, Salvador F, and Lewis, Roger A	279
Effect of Para-aminohippuric Acid on Sodium Thiosulfate Determinations in Renal Clearance Studies	Elliott, Stuart R, II, and Scott, H William, Jr	213
Elliott, Stuart R, II	Effect of Para-aminohippuric Acid on Sodium Thiosulfate Determinations in Renal Clearance Studies	213
Estradiol, Effect on Female Albino Rat		279
Fisher, A Murray	The Clinical Manifestations of the Severe Form of Diphtheria	297
Folic Acid Antagonists, Observations on the Effects of on Embryonated Eggs		275
Furstenberg, Frank F	(See Gay, Leslie N)	356
Gale, Ernest Frederick	The Nitrogen Metabolism of Gram-positive Bacteria	119
Gay, Leslie N	Comparative Study of Antihistamine Substances III Clinical observations	356
Gay, Leslie N	(See Landau, S Walter)	330, 343
Genecin, Abraham	(See Genest, Jacques)	429
Genest, Jacques	Renal Function Before and After Surgical Resection of Coarctation of the Aorta	429
Glutamic Acid, Intracellular Utilization of		134
Griswold, H E	(See Bing, R J)	439
Handelsman, J C	(See Bing, R J)	439
Harvey, A, McGehee	(See Roeder, Kenneth D)	600

Hemorrhagic Diathesis, Circulating Anticoagulant as Cause of in Man	288
Herman, Nathan B (See Gay, Leslie N)	356
Intracellular Utilization of Glutamic Acid and Its Inhibition by Certain Antibacterial Agents	134
Irradiation of Lymphoid Tissue in Diseases of the Upper Respiratory Tract Proctor, Donald F, Polvogt, Leroy M, and Crowe, Samuel J	383
Iso par, Therapeutic Evaluation of in Otitis Externa	225
Johns Hopkins Medical Society, Proceedings of the Meeting	109
Kattus, Albert A (See Genest, Jacques)	429
Koppanyi, Theodore Acetylcholine as a Pharmacological Agent	532
Krop, Stephen (See Nachmansohn, David)	493
Kuffler, S W (See de N6, R Lorente)	529
Landau, S Walter Comparative Study of Antihistamine Substances I Introduction and Dale Experiments	330
II Activity in Vivo against Histamine Intoxication and Anaphylactic Shock of Guinea Pigs	343
Landau, S Walter (See Gay, Leslie N)	356
Lewis, Roger A (See Nyda, Morton J)	279
Lobotomy, Prefrontal for the Relief of Intractable Pain	229
Lymphoid Tissue, Irradiation of	383
Marrazzi, Amedeo S (See Welsh, John H)	580
Marriott, Henry J L (See Landau, S Walter)	343
Meeting of the Johns Hopkins Medical Society	109
Morgan, Herbert R (See Wagley, Philip F)	275
Morse, William I, II (See Conley, C Lockard)	288
Nachmansohn, David The R6le of Acetylcholine in Conduction	463
Nature of Penicillin Sensitivity in Staphylococcus Aureus	154
Nelson, William H (See Gay, Leslie N)	356
Nerve Cell A Study of Experimental Poliomyelitis in the Spinal Cord	1
Newman, Elliot V (See Genest, Jacques)	429
Nitrogen Metabolism of Gram-positive Bacteria, The Gale, Ernest Frederick	119
Nyda, Morton J The Effect of Ovariectomy and Physiologic Doses of Estradiol upon Body Weight, Linear Growth, and Fat Content of the Female Albino Rat	279
Observations on the Effects of Folic Acid Antagonists, Folic Acid, Liver Extract and Vitamine B ₁₂ on Embryonated Eggs A Preliminary Report Wagley, Philip F and Morgan, Herbert R	275
Otenasek, Frank J Prefrontal Lobotomy for the Relief of Intractable Pain	229
Otitis Externa, Therapeutic Evaluation of Iso-par in	225
Ovariectomy, Effect on Female Albino Rat	279

Pain, Intractable, Prefrontal Lobotomy for the Relief of	229
Para-aminohippuric Acid, Effect of on Sodium Thiosulfate Determinations in Renal Clearance Studies	213
Paracolon Bacilli, The Cultural Differentiation of	367
Paralysis A Study of Experimental Poliomyelitis in the Spinal Cord	1
Parson, John W (See Gay, Leslie N)	356
Payne, Torrence P B Antibody Formation in Alloxan Diabetes	326
Penicillin Sensitivity in Staphylococcus Aureus	154
Physiological Studies in Congenital Heart Disease VI Adaptation to Anoxia in Congenital Heart Disease with Cyanosis Bing, R J , Vandam, L D , Handelsman, J C Campbell, J A , Spencer, R , and Griswold, H E	439
Poliomyelitis, A Study of Experimental Poliomyelitis in the Spinal Cord	1
Polvogt, Leroy M (See Proctor, Donald F)	383
Prefrontal Lobotomy for the Relief of Intractable Pain Otenasek, Frank J	229
Proceedings of the Meeting of the Johns Hopkins Medical Society	109
Proctor, Donald F Irradiation of Lymphoid Tissue in Diseases of the Upper Respiratory Tract	383
Quaternary Ammonium Ions and Sodium Ions in Nerve Physiology de N6, R Lorente	497
Rathbun, Howard K (See Conley, C Lockard)	288
Renal Clearance Studies, Effect of Para-aminohippuric Acid on Sodium Thiosulfate Determinations in	213
Renal Function Before and After Surgical Resection of Coarctation of the Aorta Genest, Jacques, Newman, Elliot V , Kattus, Albert A , Sinclair-Smith, Bruce, and Genecin, Abraham	429
Revilla, Antonio Gonzalez Differential Diagnosis of Tumors at the Cerebellopontile Recess	187
Robinson, James E , Jr (See Conley, C Lockard)	288
Roeder, Kenneth D Effect of Anticholinesterases and Related Substances on Nervous Activity in the Cockroach	587
R6le of Acetylcholine in Conduction, The Nachmansohn, David	463
Schaub, Isabelle G The Cultural Differentiation of Paracolon Bacilli	367
Scott, H William, Jr (See Elliott, Stuart R , II)	213
Sickling Phenomenon, Sulfhydryl Compounds and the	176
Sinclair-Smith, Bruce (See Genest, Jacques)	429
Spencer, R (See Bing, R J)	439
Staphylococcus Aureus, Penicillin Sensitivity in	154
Stetson, Chandler A , Jr (See Thomas, Lewis)	176
Sulfhydryl Compounds and the Sickling Phenomenon A Preliminary Report Thomas, Lewis and Stetson, Chandler A , Jr	176
Symposium on the Physiology of Acetylcholine Held at the Medical Division, Army Chemical Center, Md , 21 April 1948	463
Therapeutic Evaluation of Iso-par in Otitis Externa Walker, James S	225
Thomas, Lewis Sulfhydryl Compounds and the Sickling Phenomenon	176

Tumors at the Cerebellopontile Recess	187
Vandam, L D (See Bing, R J)	439
Virus, the Nerve Cell, and Paralysis, The A Study of Experimental Polomyelitis in the Spinal Cord Bodian, David	1
Wagley, Philip F Observations on the Effects of Folic Acid Antagonists, Folic Acid, Liver Extract and Vitamine B ₁₂ on Embryonated Eggs A Preliminary Report	275
Walker, James S Therapeutic Evaluation of Iso-par in Otitis Externa	225
Welsh, John H Concerning the Mode of Action of Acetylcholine	568
Winkenwerder, Walter W (See Gay, Leshe N)	356
Ziegler, Robert F The Cardiac Mechanism during Anesthesia and Operation in Patients with Congenital Heart Disease and Cyanosis	237